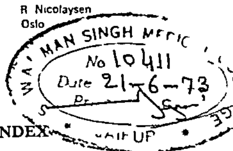
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From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

The Influence of Chlorpromazine on the Uptake of Biogenic Amines by Rat Mast Cells in vitro

By

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Abstract

FRISK HOLMBERG M and B UVNÄS *The influence of chlorpromazine on the uptake of biogenic amines by rat mast cells in vitro* Acta physiol scand 1972 *86* 1—11

Rat mast cells were incubated with ^{14}C-5 hydroxytryptamine (5 HT) ^{14}C-dopamine (DA) ^{14}C noradrenaline (NA) or ^{14}C histamine (Hi) The influence of amine concentrations temperature and metabolic inhibitors indicate that carrier mediated transport mechanisms are involved in the uptake of the amines Chlorpromazine (CPZ) in concentrations of 0 1—10 μM inhibited the uptake of 5 HT DA and NA competitively and reduced the uptake of Hi Ca^{++} decreased the effects of CPZ The results suggest that Ca^{++} may play an important part in the mechanism of action of CPZ

Rat mast cells contain 20—25 μg per million cells of histamine (Hi) and 1 μg per million cells of 5 hydroxytryptamine (5 HT) (for references see Diamant and Lowry 1966) and they are able to take up these amines both *in vivo* and *in vitro* (Furano and Green 1964 Cabut and Hægermark 1966 Jansson 1970) Dopamine (DA) and noradrenaline (NA) are not found endogenously in rat mast cells but the cells can take up and store also these two amines (Enerback and Haggendal 1970 Heisler in press)

Shanes (1958 a, b) termed drugs like chlorpromazine (CPZ) membrane stabilizers because of their property to reduce the permeability of various biological membranes CPZ for example protects red cells platelets and protozoa against hypotonic lysis (for reviews see Seeman 1966 Domino *et al* 1968 Langslet and Øye 1970) and prevents the active transport of biogenic amines *in vitro* (Axelrod *et al* 1961 Dengler *et al* 1961 Euler and Lishajko 1965 Seeman 1966)

Recently it was shown that CPZ increased the resistance of rat mast cells to hypotonic lysis (Frisk Holmberg 1971) Based on this finding and the previous reports the present study was carried out to investigate the effect of CPZ on the uptake of 5 HT DA NA and Hi by rat mast cells *in vitro*

Methods

Mast cells were isolated from the abdominal and pleural cavities of male Sprague Dawley rats by Ficoll density gradient centrifugation (according to Uvnas and Thon 1959) suspended and counted as previously described (Frisk Holmberg 1971)

Incubation procedure Between 300 000—400 000 mast cells were transferred from the stock suspension to plastic tubes and unless otherwise stated preincubated for 5 min at 37 C with or without CPZ After centrifugation the supernatants were decanted and the precipitated cells resuspended in 1 ml of incubation solution (154 mM NaCl 2.7 mM KCL 0.9 mM $CaCl_2$ and 10 % (v/v) Sorensen buffer pH 7.0 (Na_2HPO_4+KH_2PO_4 67 mM) The incubation fluid had been equilibrated with 5 % carbondioxide in oxygen to incubation temperature and contained drugs labelled amines ions etc to be tested A homogenous suspension was obtained by careful turning of the tubes which were then incubated for 10 min (in time course experiments up to 2 h) in a metabolic shaker at 37 C To allow the study of the influence of Ca^{++} and Mg^{++} on the amine uptake Ca and Mg^{++} free solutions were used until the respective ion was added to the incubation medium as described by Frisk Holmberg (1971)

Incubation was stopped by rapid transfer of the test tubes to an ice chilled water bath of 0—4 C The samples were then centrifuged (at 4 C at 360×*g* for 10 min) and washed 3 times (at 4 C) in 1 or 2 ml of incubation solution to which 0.01 % human serum albumin had been added to decrease spontaneous release of histamine During these procedures 15±3.6 % (n = 42) of total endogenous histamine content was released

The precipitated cells were lysed in 1 ml deionized-distilled water frozen thawed and shaken 3 times After centrifugation (2000×*g* for 10 min) aliquots from the supernatants and sediments were taken for measurements of radioactivity and endogenous histamine By the lysis procedure the total amounts of endogenous histamine and of exogenous amine taken up were released

Measurement of radioactivity Radioactivity in supernatants washes and sediments was measured in a Tri Carb liquid scintillation spectrometer model 3375 using the scintillation system described previously (Frisk Holmberg 1971) The radioactivity in the washes declined progressively towards background values The radioactivity of the washed sediments were 8 (5 HT NA DA) to 10 (Hi) times the background

Determination of histamine was carried out by the fluorimetric method of Shore *et al* (1959) as described by Frisk Holmberg (1971)

Control of metabolite formation during incubation Ascending paper chromatography was carried out on a Whatman no 1 paper Aliquots of lysed cell material were run together with amine references overnight dried and stained with ninhydrin Cut pieces of the dried chromatograms were placed in scintillation fluid and the radioactivity measured The following solvents systems were used Isopropanol ammonium hydroxide water (20 1 2) for 5 HT n butanol acetic acid water (120 30 50) for Hi NA and DA

Each chromatogram showed only one spot. This spot corresponded to the reference spot and also contained all the radioactivity on the paper These findings were taken as evidence that no amine metabolites were formed in measurable amounts during incubation Moreover neither monoamine oxidase nor diamine oxidase are found in rat mast cells (Green 1966)

Amine stability during incubation The native fluorescence of DA and NA was measured after 10 30 60 120 min of incubation at pH 6.0 7.0 7.5 (37 C) In samples incubated at pH 7.5 (n = 3) a decrease in the fluorescence of both NA and DA was found 10.2±5.0 % (NA) and 15.0±3.9 % (DA) respectively after 10 min incubation and 40.0±1.1 % (NA) and 50.2±6.1 % (DA) after 60 min incubation. After incubation for 10 min pH 7.0 (n = 3) no significant decrease in fluorescence was found In samples incubated at pH 7.0 (n = 3) a decrease in amine fluorescence was found 3.5±2.6 % for NA and 8.0±0.5 % for DA after 60 min and 13.1±3.6 % (NA) and 14.0±1.6 % (DA) after 120 min incubation This reduction in fluorescence was prevented by adding ascorbic acid to the incubation medium Therefore in time course and pH experiments (see Results) ascorbic acid 10 µg/ml was added to the incubation medium The stability of 5 HT was measured by spectrophotofluorimetry according to Weissbach (1961) and that of histamine by fluorimetry according to Shore *et al* (1959) No measurable change in the content of these amines under the circumstances above was observed

Influence of Ca^{++} on the uptake of (^{35}S) CPZ to mast cells 500 000 cells were incubated at 22 C in preweighed plastic tubes with (^{35}S) CPZ in concentrations from 5 to 20 µM in a Ca^{++} free or Ca^{++} (1—4 mM) containing medium at pH 7.0 (154 mM NaCl 2.7 mM KCl and 10 % (v/v) 50 mM Tris buffer Incubation volume was 100 µl After 5 min the incubation samples were centrifuged (360×*g* for 10 min) and the supernatants removed with a micropipette The error of the method was 1.8±0.6 (n = 8) Uptake of (^{35}S) CPZ was estimated from the decrease of radioactivity in the incubation medium The tubes were then

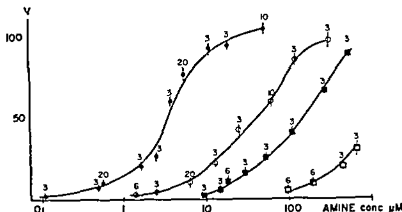


Fig 1 Influence of extracellular amine concentration on uptake of amines by mast cells 10 min incubation at 37 C pH 7 0 Ordinate Uptake rates (V) expressed as pmole/min/10^6 mast cells (mean ± S D) Abscissae Extracellular amine concentration µM Figures denote number of experiments ●———● 5 HT ○———○ DA ■———■ NA □———□ Hi

reweighed (this gave weight of remaining drop the weight of the mast cells being neglected) and samples resuspended and lysed in 1 ml 0 1 N NaOH and centrifuged at 400 × g for 10 min Aliquots were taken from all fractions for radioactivity and histamine determinations The amount of radioactivity regained after lysis of cells in 1 ml of 0 1 N NaOH was corrected for radioactivity in the remaining drop

Calculations Uptake rates are expressed as pmoles of amine base/min/10^6 mast cells (mean ± S D) Uptake was measured after 10 min incubation during which period the uptake was linear with time (see Fig 6) As far as possible the effects of metabolic inhibitors temperature and ions were investigated on the same uptake rates of the amines

Materials

Isotopes (^{35}S) chlorpromazine (11 4 mCi/mmol) (CPZ) was kindly supplied by AB Leo Helsingborg Sweden 5 hydroxytryptamine 2 C^{14} binoxalate (11 46 mCi/mmol) (5 HT) was purchased from New England Nuclear Corp Boston Mass USA and dopamine (ethylamine 2 C^{14}) hydrochloride (55 mCi/mmol) (DA) L-noradrenaline (carbinol C^{14}) bitartrate (57 mCi/mmol) (NA) and histamine (ring 2 C^{14}) dihydrochloride (54 3 mCi/mmol) (Hi) from the Radiochemical Centre Amersham England

Nonradioactive reagents Amines enzyme inhibitors and other analytical grade reagents and chemicals were obtained from regular commercial sources Drug solutions were freshly prepared in deionized distilled water

Results

I *The uptake of 5 HT DA NA and Hi*

I 1 *Influence of external amine concentration* (Fig 1) All the four amines studied were taken up by the mast cells Marked differences were noted in the uptake rates of the amines The uptake of 5 HT and DA increased with increasing extracellular amine concentrations approaching saturation level The maximal uptake rate was similar for 5 HT and DA and occurred at an extracellular amine concentration around 50 µM of 5-HT around 200 µM of DA The uptake rate of NA reached the

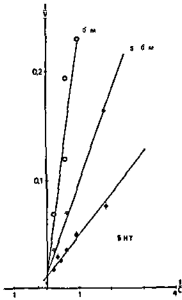


Fig 2

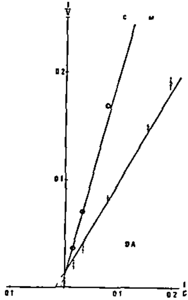


Fig 3

Fig 2 Double reciprocal plot of 5 HT uptake The initial rate of uptake (V) pmol/min/10^6 mast cells (ordinate) versus extracellular amine concentration (C) μM (abscissae) Regression lines were calculated according to the method of least squares Means and S D of 4 expts Mast cells were incubated for 10 min 37 C pH 7 0 in a medium with or without CPZ 5 10^{-6} -10^{-5} M Maximal rate of uptake 100±11 1 pmol/min/10^6 mast cells Affinity for uptake 3 3 μM Control uptake ●————●

Fig 3 Double reciprocal plot of DA uptake Text and symbols see figure legend 2 Maximal rate of uptake 83±8 3 pmol/min/10^6 mast cells Affinity for uptake 67 μM Control uptake ○————○

same values as those of the former amines but a similar degree of saturation was not obtained at the highest NA concentrations used The Hi uptake rate increased with increasing extracellular amine concentrations without levelling off within the concentration range used (1—600 μM) Addition of glucose 0 6 or 2 7 mM to the incubation medium did not influence the uptake velocity of the amines

Double reciprocal plots (Fig 2 3) indicated that the maximal uptake rates of 5 HT and DA were almost similar but that the affinity for uptake varied This was thus greater for 5 HT being approximately 20 times the affinity for DA A similar plot of NA uptake (Fig 4) revealed that the affinity for this uptake was 60 times lower than the affinity for 5 HT uptake

I 2 *Influence of incubation time and temperature* As seen in Fig 6 the amine uptake increased linearly with time for at least 1 h if the following extracellular amine concentrations were used 0 56 μM of 5 HT 6 5 μM of DA 19 μM of NA and 99 μM of Hi At higher extracellular amine concentrations or after longer incubation periods (than those in Fig 6) a gradual retardation of the amine uptake was found The amine concentrations studied gave constant uptake rates during at least the first 10 min incubation period

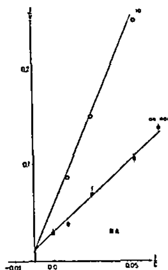


Fig 4

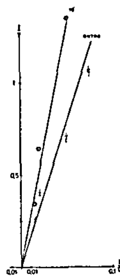


Fig 5

Fig 4 Double reciprocal plot of NA uptake Text and symbols see figure legend 2 Maximal rate of uptake 99±6.5 pmol/min/10⁶ mast cells Affinity for uptake 182 μM Control uptake ■———■

Fig 5 Double reciprocal plot of Hi uptake Text and symbols see figure legend 2 Control uptake □———□

Increase in temperature (5° to 45° C) increased the uptake rates of 5 HT DA and NA (Fig 7)

I 3 *Influence of pH* Variations of the pH between 6 0—7 5 had no significant effect on the uptake rates of 5 HT NA and Hi The uptake rate of DA was faster at lower pH values (Table I)

I 4 *Influence of metabolic inhibitors* N-ethylmaleimide ninhydrin 2-4 dinitrophenol and ouabain in concentrations 10^{-4}—10^{-3} M decreased the uptake rates of 5 HT DA and NA 5 HT uptake was the most sensitive to these inhibitors (see Table II)

I 5 *Influence of Ca and Mg⁺⁺* Ca^{++} stimulated the uptake of 5 HT and DA the uptake rates being almost doubled in the presence of 1 mM Ca The uptake rates of NA and Hi were not significantly influenced by this concentration of Ca^{++} (Table III) whereas the presence of 4 mM Ca^{++} seemed to reduce the uptake rates of both NA and 5 HT and DA Mg^{++} (1—4 mM) did not influence the uptake of 5 HT and DA

II *The influence of CPZ on amine uptake*

II 1 CPZ in concentrations 10^{-7}—10^{-5} M depressed the uptake rates of the four amines studied but to a varying degree The reduced uptake of the amines re

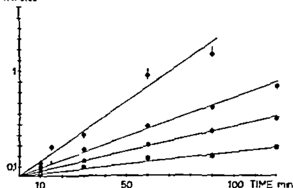
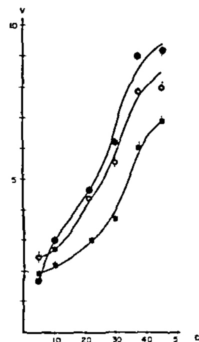


Fig 6 Influence of incubation time on amine uptake Ordinate Uptake nmol/10^6 mast cells Abscissae Time min Means ± ranges of 2 expts ●——● 5 HT ○——○ DA ■——■ NA and □——□ Hi



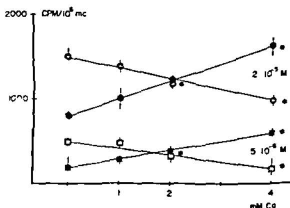


Fig 7 Fig 8

Fig 7 Influence of temperature on the uptake rates of 5-HT 0 56 μM ●——● DA 6 5 μM (○——○) NA 19 μM (■——■) Ordinate Uptake rate (V) pmol/min/10^6 mast cells Abscissae Temp C Values represent mean ± ranges of 2 expts. Cells were incubated at the stated temperature for 10 min

Fig 8 Influence of Ca on the uptake of (^{35}S)-CPZ to mast cells. Ordinate cpm/10^6 mast cells (mc) Abscissae Ca concentration in incubation medium. Values are mean ± S D ($n = 4$) *Significant differences between values from a Ca containing compared to a Ca^{++} free incubation are indicated by* • ($p < 0.05$) Cpm in incubation supernatant ●——● after incubation with 2 10 M (^{35}S)-CPZ ■——■ after incubation with 5×10^{-6} M (^{35}S)-CPZ Cpm in supernatant after lysis of cells treated with 2×10 M (^{35}S) CPZ ○——○ and with 5×10^{-6} M (^{35}S)-CPZ □——□

TABLE I Mast cells were incubated at different pH with 5 HT 0 56 μM DA 6 5 μM NA 19 μM or Hi 99 μM for 10 min Uptake rates expressed in pmol/min/10^6 mast cells (Means ± S D) Figures with asterises indicate values significantly higher ($p < 0\ 01$) than values at pH 7 5 Figures in brackets denote number of experiments

Amine	Uptake rate pH			
	6 0	6 5	7 0	7 5
5 HT	9 4±1 0(6)	8 5±2 1(6)	9 7±0 9(6)	9 4±0 8(6)
DA	9 3±0 1(6)	10 1±1 2(6)*	8 3±0 7(6)	[illegible] 7±0 5(6)
NA	11 0±4 3(3)	10 0±0 3(3)	8 8±2 1(6)	9 4±0 2(3)
Hi	4 1±0 8(4)	3 9±1 0(4)	3 5±0 6(4)	4 0±1 5(4)

mained linear with time during the period studied The uptake of 5 HT DA and NA could be almost totally inhibited and the uptake of Hi was reduced The uptake velocities were plotted against extracellular amine concentrations in a double reciprocal manner as shown in Fig 2—5 A competitive antagonism was found between CPZ and the amines 5 HT DA and NA Preincubation of the cells with CPZ was not necessary for full inhibitory effect The blocking effect of CPZ could be partly abolished by repeated washing of the cells in the incubation medium The uptake rates (5 HT DA NA and Hi) in cells treated with CPZ 10^{-5} M were restored to approximately 50 % after one wash and to almost 80 % after two washes When cells were treated with CPZ 5×10^{-6} M one wash restored the uptake rates to 80 %

II 2 *Influence of Ca and Mg on the effect of CPZ* The presence of Ca^{++} (2—4 mM) in the medium counteracted the inhibitory effect of CPZ on amine uptake (see Table III) The counteracting effect of Ca was observed already in the presence of 2 mM Ca and was maximal at 4 mM (increase of the Ca concentrations to 8 mM produced no further effect) if concentrations of CPZ were used which gave 50 % inhibition of amine uptake Low concentrations of CPZ (2×10^{-7} M) were more sensitive to the effect of Ca whereas higher concentrations (10 M) of CPZ were less influenced Mg (1—4 mM) did not influence the effect of CPZ on 5 HT and DA uptake

TABLE II Mast cells preincubated with inhibitor for 15 min before addition of labelled amine Values (means and ranges of 2 expts) are expressed as inhibition of control uptake rate during 10 min incubation

Ami	N ylm l im d		Ni hyd		2 4Di lt ph l		O b i	
	10^{-4}M	10^{-3}M	10^{-4}M	10^{-3}M	10^{-4}M	10^{-3}M	10^{-4}M	10^{-3}M
5 HT 0 56 μM	75 2 2	95 ± 3 1	40 ± 2 2	90 ± 4 6	67 ± 2 2	90 ± 0 5	32 ± 5 6	60 0 5
DA 6 5 μM	61 ± 3,0	85 ± 1 6	20 ± 1 1	88 ± 2 1	60 ± 6 0	80 ± 2,0	15 ± 1 1	50 ± 1 2
NA 19 μM	45 0 5	50 2,0	20 1 2	58 ± 1 1	41 ± 3 6	60 ± 2 6	21 ± 3,0	42 6 1

TABLE III Mast cells in a Ca⁺⁺ free and Ca⁺⁺ (1 – 4 mM) containing medium (pH 7 0) pre[…] min/10 mast cells (mean ± S D) I indicates % inhibition of control uptake rate (mea[…] mated by Student s t test) changes of I in a Ca⁺⁺ containing medium compared to […] by asterises $*p < 0\ 05$ $**p < 0\ 01$ Statistically significant changes of V in a Ca⁺[…]

Amine Conc µM	CPZ Conc M	Ca⁺⁺ Conc V	I	1 mM V
	–	8 8 ± 1 0(5)		18 0 ± 5 6(5)●
5 HT	2×10^{-7}		27 0 ± 5 6	
0 56	2×10^{-6}		50 0 ± 10 1	
	10^{-}		90 0 ± 0 1	
	–	8 8 ± 0 7(5)		15 6 ± 1 0(5)●
DA	$2\times10^{-}$		30 0 ± 5 6	
6 5	2×10^{-6}		48 1 ± 1 3	
	10^{-}		75 0 ± 3 7	
	–	14 2 ± 0 6(3)		11 9 ± 0 3(3)
NA	$2\times10^{-}$		43 0 ± 5 3	
19	2×10^{-6}		52 8 ± 3 6	
	10^{-5}		62 7 ± 8 9	
	–	*4 5 ± 0 3(3)*		*4 1 ± 1 0(3)*
Hi	2×10^{-7}		–	
99	2×10^{-6}		20 1 ± 5 5	
	10^{-}		59 8 ± 10 5	

III *Influence of Ca on the uptake of (^{35}S) CPZ by mast cells* On the incubation of mast cells in (S) CPZ containing solutions the radioactivity of the incubation fluid declined indicating an uptake of CPZ by the cells In the presence of Ca (2 or 4 mM) the uptake was reduced the radioactivity in the incubation fluid being higher the activity in the lysed cell material being lower (Fig 8) than in the absence of Ca At an extracellular concentration of 2×10^{-4} M CPZ 10^{6} mast cells took up $1\ 18\pm0\ 14\times10^{-8}$ (n = 4) mol of CPZ This uptake was reduced to $0\ 68\pm0\ 23\times10^{-8}$ mol (n = 4) in the presence of 4 mM Ca

Discussion

The present observations confirm that mast cells *in vitro* are capable of taking up and storing not only the endogenously occurring mast cell amines Hi and 5 HT but also DA and NA which do not occur in rat mast cells *in vivo*

The uptake rates for 5 HT DA and NA increased with rising extracellular amine concentration to approach a maximum for 5 HT around 50 µM for DA above 200 µM The uptake of NA and of Hi did not show any similar saturation level within the concentration ranges used (Fig 1) The uptake rates for 5 HT DA and NA increased with rising temperature (Fig 7) Enzyme inhibitors (n ethylmaleimide ninhydrin 2-4-dinitrophenol and ouabain) reduced the uptake rates and it has also been shown that these amines compete for uptake in mast cells (Heisler in press)

cubated with CPZ before exposure to labelled amine V indicates uptake rate in pmol/ ±S D) Figures in brackets indicate number of experiments Statistically significant (esti Ca++ free (NA Hi) and to a 1 mM Ca++ containing medium (5 HT DA) are indicated containing compared to a Ca++ free medium are indicated by ● p < 0 01

	2 mM		4 mM	
I	V	I	V	I
	13 6±3 6(5)		8 0±0 1(5)	
24 3±3 6		10 5±5 6		8 0±1 1
66 2±2 1		41.2±1 9*		3? 1±2.2 *
90 1±3 6		93 0±1 1		84 3±2.2*
	8 5±0 1(5)		7 9±1 1(5)	
34 5±1 1		32 7±2 7		14 6±1 7 *
48.2±5 6		46 8±0 8		25 7±2 5
86 1±4 6		64 9±3 3*		70.2±1 9
	12 5±0 5(3)		10 6±0 4(3)	
20 5±2 6		8 9±1 6		1 3±2.2**
48 7±3 6		20 4±3 1		15 3±? 6*
56.2±5 3		35 0±6 6		3? 1±3 1*
	3 6±0 9(3)		5 0±1 5(3)	
—		—		—
13 1±6 0		5 4±2 0 *		9 9±? 0*
56 6±10 0		30 0±9 1*		14 9±4 0**

The above mentioned data point to carrier mediated energy requiring transport of 5 HT NA and DA

As to the influence of pH and Ca++ minor differences were observed While the uptake rates for 5-HT and NA did not vary significantly within the pH range 6 0—7 5 the uptake rate for DA was considerably lowered at pH 7 5 Differences in the degree of dissociation of the amines do not explain the reducing effect of pH on DA uptake since all amines used have similar pKa values namely NA 9 78 Hi 9 80 5 HT 9 80 DA 10 63 (Rapport *et al* 1948 Lewis 1954 Long 1961) The presence of Ca stimulated the uptake of 5 HT and DA but did not seem to have any effect on the uptake of NA The significance of these minor differences regarding the influence of pH and Ca++ on the uptake of these amines is not clear Whether the differences indicate the existence of separate carrier systems for the amines or only reflect minor differences in their transport systems cannot be answered at present.

The uptake of Hi seemed to follow a pattern different from those of 5 HT DA and NA It did not show any saturation level and was not significantly influenced by enzyme inhibitors (Cabut and Hægermark 1966) But since also Hi can compete with the uptake of the other amines (Heisler in press) simple passive diffusion may not suffice to explain the uptake of Hi

In concentrations of 4 mM Ca seemed to reduce amine transport A possible explanation for this finding could be a physical type of action of Ca ions rendering the membrane less permeable (Manery 1966)

In order to find out if saturation of the granule binding sites could be the reason

for the retarded uptake rate at higher extracellular amine concentrations or at prolonged amine uptake the amounts of amines taken up in one hour by the mast cells were calculated Assuming an uptake rate of 0 1 nmol/min/10^6 mast cells, which is around the maximal uptake rate observed the total amine uptake would amount to 6 nmol/10^6 mast cells The storage capacity for biogenic amines (Hi, NA, DA and 5 HT) of rat mast cell granules is approximately 1000 nmol/mg granules (Uvnas *et al* 1970, Bergendorff and Uvnas 1972) 10^6 mast cells contain approximately 220 nmol of Hi and 10 nmol of 5 HT and have a dry weight of approximately 0 5 mg The granule fraction (protein heparin complex) accounts roughly for 80 % of this value (Diamant and Lowry 1966) This would give a granule dry weight of approximately 0 40 mg with an amine binding capacity of 400 nmol This means that only about 45 % of the granule binding capacity seems to be utilized for the binding of the normal amount of the endogenous amines In the present example at maximal uptake rate 6 nmol of amine was calculated to be taken up in 1 h, this amount would then only occupy about 3 % of the remaining binding capacity of the granules (after substraction of the endogenous amine store) This fact argues against a saturation of the binding sites in the granules as being the cause of the levelling off of the uptake rates

As demonstrated in the present experiments on mast cells and previous observations on thrombocytes CPZ is an effective inhibitor of active membrane transport processes (Stacey 1961 Seeman 1966) within the concentration range (10^{-7}—10 M) as in this study It also counteracts the lytic effect of hypotonic solutions on various cells (Seeman 1966) among them mast cells (Frisk Holmberg 1971) The unspecificity of the inhibitory action of CPZ may be connected with structural changes of the membranes caused by the drug It has been claimed to change the crystalline structure of membrane lipids with consequent changes in the protein lipid linkage in the membranes (Hubell *et al* 1969)

It has been proposed that CPZ (and local anesthetics) displace membrane bound Ca (Kwant and Seeman 1969 Suarez Kurtz *et al* 1970) and Ca bound to phospholipids *in vitro* (Dawson and Hauser 1970) Ca are known to be essential for various active transport processes in membranes Ca (1 mM) stimulated the uptake of 5 HT and DA by mast cells Higher extracellular concentrations of Ca^{++} (2—4 mM) reduced the uptake of CPZ in mast cells and reduced the inhibitory action of CPZ on the uptake of amines

It might be that the competitive inhibitory action of CPZ on the uptake of biogenic amines reflects its interference with the membrane bound Ca thereby influencing the efficacy of the amine transport mechanisms

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Inhibition by Hippurate and Probenecid of in vitro Uptake of Iodipamide and o-Iodohippurate A Composite Uptake System for Iodipamide in Choroid Plexus, Kidney Cortex and Anterior Uvea of Several Species

By

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Abstract

BARANY E H *Inhibition by hippurate and probenecid of in vitro uptake of iodipamide and o iodohippurate A composite uptake system for iodipamide in choroid plexus kidney cortex and anterior uvea of several species* Acta physiol scand 1972 *86* 12—27

Slices of kidney cortex and liver pieces of lateral choroid plexus and of ciliary body with or without iris were incubated in a potas ium rich medium containing labelled iodipamide often together with labelled o-iodohippurate Tissues were from rabbits guinea pigs monkeys (3 species) dogs cats and chicken Iodipamide at 0.5—2 μM depresses uptake of labelled iodipamide by rabbit kidney cortex without affecting uptake of o-iodohippurate Hippurate at 30 mM suppresses uptake of o-iodohippurate in all tissues tested but leaves a fraction of iodipamide uptake in all tissues and species tested The findings with o-iodohippurate uptake are compatible with a single uptake system the findings with iodipamide and the kinetics derived for this substance are incompatible with a single uptake system Iodipamide seems to be accumulated by one hippurate sensitive s stem shared with o-iodohippurate and at least one other hippurate in ensitive system This latter system usually is a minority system in kidney cortex and anterior uvea but dominates in liver and choroid plexus and in cat kidney cortex The hippurate insensitive system can be suppressed b probenecid but needs a higher concen tration than the hippurate sensitive one

There are transport systems that remove organic acids such as iodopyracet from the vitreous cavity (Forbes and Becker 1960) and from the cerebral ventricles (Pappen heimer Heisey and Jordan 1961) These have much in common with the well known hippurate transporting system in the tubules of the kidney

Sperber (1947 1949 has pointed out that detoxication mechanisms frequently collaborate with excretory mechanisms in such a manner that detoxication products are actively secreted by the kidney tubules and/or the liver The transport systems found by Becker and by Pappenheimer add another dimension substances excreted by the kidney are kept out of the eye and as foreseen by Steinwall (1961) the brain by what is in effect an extrarenal kidney But is there an extrahepatic liver too? To investigate this question one needs suitable test substances

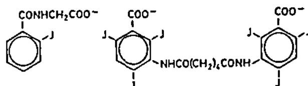


Fig. 1. The anions of o-iodohippurate (left) and iodipamide

It is well known that many substances are secreted by the kidney tubules as well as by the liver. But there are specificities. For kidney scintigraphy the old x-ray contrast medium o-iodohippurate (Hippuran®) has become popular because of its high degree of selectivity (Nordyke, Tubis and Bladh 1960). Among substances secreted by the liver the cholangiographic agents iodipamide (Biligrafin®, Cholografin®) and its congener ioglycamic acid (Bilivistan®) are remarkable for their extremely rapid secretion into the bile. Fig. 1 shows the structure of the first mentioned substances.

Since odohippurate and iodipamide are commercially available labelled with different iodine isotopes it was easy to study their simultaneous uptake *in vitro*. It then was found—in several extrahepatic tissues of several species—that iodipamide is handled by at least two different transport systems. One system is shared with iodohippurate and seems to be identical with the well known hippurate system of the kidney (which may or may not be a single one). Another possibly composite transport system for iodipamide is much less sensitive to excess hippurate. It may represent the extrahepatic liver. The present paper reports evidence for the existence of the hippurate insensitive iodipamide transport system(s).

Methods

Rabbits, mainly pigmented, were bought from various dealers. Guinea pigs were Dunkin Hartley from Åkers Krutbruk, Sweden. A few dogs, monkeys (several species) and cats were also used; they were from various sources. All the animals were kept on conventional diets and had free access to tap water. They were not starved before the experiment. The two sexes were used indiscriminately.

The animals were anesthetized with pentobarbital or urethane. The rabbits were anesthetized with urethane, one kidney and one eye removed and tissues from these incubated. About 1 h later the animal was killed after removal of the other kidney, the other eye and sometimes samples of the liver. Lateral ventricular plexus was taken after death. In smaller animals all samples were taken at the same occasion. Tissues were also removed from dogs, cats and monkeys recently killed for other purposes.

The kidneys were decapsulated and transverse horse shoe shaped slices cut to 0.3—0.5 mm thickness with a modified Stadie Riggs slicer. They were immediately put into very shallow basal incubation medium exposed to room air at 37 °C in Petri dishes. There the pieces were trimmed and cortex separated from medulla. The juxta medullary parts of cortex were also discarded. Pure medulla does not take up iodipamide.

After exactly 10 min the slices (each about 20 mg) were transferred to incubation bottles containing 5 ml fluid and 70 ml room air. The fluid was the basal incubation medium with radioactive test substance and inhibitor already present when the organ samples were put into it. The bottles were shaken at 37 °C without gassing. Special experiments had shown that gassing did not influence the results—there was less than 100 mg of tissue in each bottle.

After the first 20 min of incubation of rabbit kidney cortex tissue/medium ratios are still increasing. Of more interest is the time-dependence of relative uptake in partly inhibited slices. In the presence of 10 μM probenecid, the uptake expressed as per cent of that in uninhibited slices rose for the first 15—20 min but reached a plateau between 20 and 30 min both for

iodohippurate and for iodipamide (Fig 10) The incubation time used in the majority of experiments was 20—26 min sometimes 20—30 the time schedule was always balanced so that the average incubation time of the kidney slices was the same in all bottles Choroid plexus and eye tissue as a rule were only one piece per bottle but here incubation time was identical for all pieces

Choroid plexus from the lateral ventricles pieces of ciliary body with attached ciliary processes or whole anterior uvea (guinea pig rabbit) were as a rule put directly into the incubation bottle but sometimes they were kept in the Petri dishes for a few minutes This seemed to make no difference One rabbit yielded 4 pieces of plexus

After incubation the tissue pieces were fished out of the bottle blotted on filter paper and rapidly weighed on very thin Al foil on a torsion balance The samples were transferred on the foil to plastic tubes dried at 80 C over night and counted in a Packard Tri Carb with a γ spectrometry attachment Medium samples were put onto pieces of cellulose sponge weighing about 15 mg and then treated as tissue samples

In order to correct for unspecific uptake every run (with few exceptions) contained a totally inhibited bottle with 12 6 mM non radioactive iodipamide (0 2 ml Biligrafin forte® to 5 ml medium) Tissue/medium ratios of 0 64 ± 0 03 (S E) for iodipamide and 0 91 ± 0 02 (S E) for iodohippurate (see Table II) were obtained with kidney cortex in this bottle and agreed reasonably well with ratios found for tissues incubated at 0 C tissue/medium ratio 0 797 ± 0 021 (S E) for iodipamide 0 649 ± 0 019 (S E) for iodohippurate 12 kidney cortex slices from the same rabbit accumulating both compounds simultaneously at 0 C each slice counted as a unit Individually obtained unspecific uptake figures were always deducted from the uptake figures in other bottles if the contrary is not stated If no individual figures were available average figures were used

Per cent uptake figures were always calculated with the control uptake of the same animal as denominator

Solutions

All the salts used were *pro analysi*

The basal incubation solution had the following composition in mM NaCl 102 KCl 37 $MgSO_4$ 1 16 $CaCl_2$ 2 5 Na_2HPO_4 2 44 KH_2PO_4 0 58 It was adjusted to pH 7 4 with a little NaOH or HCl after the addition of 1 g/liter of glucose

In some experiments part of the chloride was exchanged against hippurate anions The hippurate was recrystallized Eastman Kodak or Schuchardt In many experiments 1 ml of isotonic non radioactive NaI solution was added to 100 ml of the incubation fluid This is sufficient to saturate the I transport system in the eye and choroid plexus (Becker 1961 a b)

Radioactive compounds

Iodipamide and o-iodohippurate tagged with one of the two iodine isotopes ^{131}I or ^{125}I respectively were present together in the incubation medium in very low chemical concentration Sometimes they were used singly Iodipamide and most of the iodo-hippurate were obtained from the Radiochemical Centre Amersham Some iodohippurate came from AB Atomenergi Studsvik Sweden The compounds were not purified before use Control experiments with the addition of non radioactive sodium iodide showed that uptake of inorganic activity into the slices was a negligible source of error In one set of experiments ^{131}I Rose Bengal from Amersham was used

Biligrafin ampoules 1 ml test ampoules of Biligrafin forte® (372 mg/ml iodipamide) were kindly donated by Schering A G Berlin through their Swedish distributors Schering also kindly disclosed the composition of the solvent A control solution of the Biligrafin solvent was made and tested It had no inhibitory effect

Results

I Complexity of the iodipamide accumulating system in rabbit kidney cortex

Fig 2 shows a key observation Rabbit kidney slices were allowed simultaneously to accumulate iodipamide and o-iodohippurate labelled with different iodine isotopes. Varying concentrations of cold iodipamide were present in the medium Each of 11 animals contributed two cortex slices at every concentration one was incubated for 20 the other for 30 min It is seen that the relative uptake of labelled iodipamide

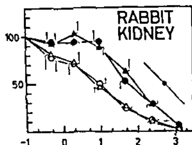


Fig 2

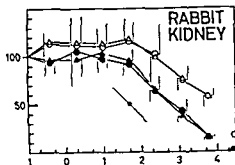


Fig 3

Fig 2 Iodipamide depresses the uptake of iodipamide before that of o-iodohippurate. Rabbit kidney cortex 11 animals 2 control bottles 1 at all other concentrations 2 slices per bottle 1 slice left for 20 the other for 30 min Abscissae log μM iodipamide ordinates tissue/medium ratio in percent of that in control slices from the same animal Open symbols iodipamide filled symbols o-iodohippurate Circles 20 min incubation triangles 30 min Bars 2 S E Control tissue/medium ratios for iodipamide 20 min 14 4±1 1 30 min 17 8±1 4 for o-iodohippurate 20 min 8 15±0 58 30 min 8 97±0 69 (n = 22 statistical unit one slice)
Concentration of labelled o-iodohippurate 0 14 μM All figures corrected for unspecific uptake
The isolated line shows the single system slope

Fig 3 Hippurate depresses the uptake of o-iodohippurate further than that of iodipamide Rabbit kidney cortex 12 animals Abscissae log μM hippurate Control tissue/medium ratios for iodipamide 20 min 16 4±1 2 30 min 21 1±1 5 for o-iodohippurate 20 min 8 21±0 47 30 min 9 01±0 48
Concentration of labelled iodipamide 0 1 μM of labelled o-iodohippurate 0 14 μM
In other respects the legend for Fig 2 holds

starts to be significantly depressed at lower concentrations of cold iodipamide than are required for the depression of o iodohippurate uptake At 1250 μM iodipamide however the uptake of both test substances is deeply inhibited

If iodipamide and o iodohippurate had been taken up by only one common system both uptakes would have been equally depressed by any competitor (Barany 1971) Moreover from simple Michaelis Menten kinetics one would expect a certain slope of the curve around the 50 % point (from 91 to 9 % in 2 log units) The figure shows that the slopes of the iodohippurate uptake curves agree reasonably well with the single system slope but that the iodipamide uptake curves are distinctly less steep which indicates the presence of more than one affinity constant a composite system Such slope indicating lines will appear in several figures in this paper

In an attempt at a more quantitative evaluation it was assumed as a first approximation that there are two independent Michaelis Menten type uptake systems for iodipamide with different affinities 1/k for iodipamide and responsible for different parts Q of the total maximum tissue/medium ratio reached at vanishing iodipamide concentrations An equation representing such a composite system (see Barany 1971 was computer fitted by a least squares program to the iodipamide uptake data of Fig 2 The curves were fitted to averages formed in different ways

TABLE I Kinetic constants derived from the data of Fig 2 The Q values indicate the percentage of the maximum tissue/medium ratio of the accumulated substance for which a transport system is responsible The k values are half saturation concentrations of iodipamide in μM The U values measure uncertainty they are root mean squares of deviations between observed averages and the computer curves Their unit is percent of maximum tissue/medium ratio Note that systems (1) and (2) accumulate iodipamide (3) and (4) o-iodohippurate but that the k values in both cases refer to iodipamide as inhibitor

Kind of average	Incubation time mins	Iodipamide uptake					o-iodohippurate uptake				
		Q_1	k	Q	k_1	U	Q_3	k_3	Q_4	k_4	U
means	20	37	0 24	63	28	2 4	93	77	7	570	3 7
means	30	43	0 73	57	35	2 7	49	63	51	130	3 8
medians	20	42	0 21	58	32	2 2	72	55	28	270	3 0
medians	30	59	1 15	41	70	1 3	105	100	−5	760	2 1
means	20+30	41	0 14	59	25	2 0	98	92	2	−500	3 0
medians	20+30	60	0 05	40	30	1 3	112	115	−12	650	2 0

All points including the 100 % point in Fig 2 were given the same weight The results are shown in the left half of Table I None of the fits was very good and the derived constants scatter a good deal Nonetheless it is clear that iodipamide is taken up by more than one system If there are only two systems they are of approximately equal importance and system (2) has 50—100 times less affinity for iodipamide than system (1) Halfsaturation of system (1) probably occurs below 1 μM of iodipamide

Exactly the same equation and procedures were applied to the iodohippurate data of Fig 2 (Table I right half) Here the computed constants Q_3 Q_4 k_3 k_4 indicate only one dominant system. Admittedly the constants for means 30 and medians 20 suggest the presence of two systems but for means 30 the two k values differ by only a factor of 2 and they might in fact be identical For the case of medians 20 the matter is somewhat more doubtful but considering the uncertainties of the measurements and the contrary evidence of the other lines of the table it cannot be taken as established that iodohippurate is transported by several systems with different affinities to iodipamide More probably it is only one This system with k_3 in the range 50—100 evidently can very well be the (2) system of iodipamide uptake with k = 25—70

In Fig 3 unsubstituted hippurate was used as inhibitor At very low concentrations of hippurate the relative uptake of iodipamide was increased It is possible but not probable that this is a real effect and not due to fortuitously low uptake at the very lowest hippurate concentration The figure shows that the relative uptake of iodipamide and of iodohippurate are depres ed by the same concentrations of hippurate but that the relative depression of iodipamide is less than that of iodohippurate At 6250 μM hippurate less than 15 per cent of o iodohippurate but more than 50 per cent of iodipamide uptake remain The slope of the o iodohippurate curve is not far from the single system slope while that of the iodipamide curve is distinctly smaller

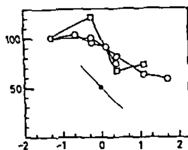


Fig 4

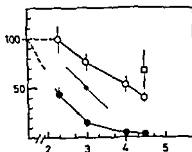


Fig 5

Fig 4 Iodipamide in low concentration depresses hippurate insensitive iodipamide uptake Kidney cortex and choroid plexus from 4 rabbits incubated 20 min in the presence of 30 mM hippurate Slices from one kidney (circles with a dot) were run at 0 05 (control) 0 25 0 55 1 30 and 2 55 μM iodipamide slices of the other kidney and pieces of choroid plexus at 0 05 0 55 2 55 12 55 and 50 μM (plexus not at 50) In each run 2 control bottles 1 at all other concentrations 2 slices per bottle Abscissae log μM iodipamide ordinates tissue/medium ratio in percent of controls Circles kidney squares plexus Control tissue/medium ratios kidney 2 12±0 12 plexus 5 95 All figures corrected for unspecifi uptake The isolated line shows the single system slope

Fig 5 Hippurate in high concentration does not abolish iodipamide uptake despite the presence of 12 5 μM iodipamide Kidney cortex and choroid plexus from 4 rabbits incubated 20 min in the presence of 12 5 μM iodipamide and 0 1 μM iodohippurate The 2 kidneys were run separately plexus together with the last one In each run 2 control bottles 1 at all other con centrations 2 slices per bottle Abscissae log μM hippurate Circles kidney square plexus Filled symbols iodohippurate open symbols iodipamide Bars 2 S E sometimes hidden in the symbols
Control tissue/medium ratios for iodipamide kidney 5 98±0 36 (n = 8)
plexus 7 13±0 95 (n = 4), for o-iodohippurate kidney 4 85±0 26 plexus 1 53±0 34
In other respects the legend for Fig 4 holds

The experiment shows that the iodipamide transporting system can be divided into one part *A* that is sensitive to hippurate and therefore probably identical with systems (2) and (3) of Table I taking up o-iodohippurate as well as iodipamide and another part *B* with very little affinity to hippurate It is not completely inhibited even by 30 mM hippurate while iodohippurate uptake is completely suppressed (the isolated points at the far right of Fig 3 derived from the experiments of Table II)

Is the high affinity part (1) of the iodipamide system of Table I and Fig 2 identical with the hippurate insensitive part *B* demonstrated in Fig 3? Fig 4 shows that part of *B* has high affinity to iodipamide For this experiment the slices were incubated in a medium containing 30 mM hippurate The tissue/medium ratios for kidney and plexus were clearly depressed already by 2 5 μM iodipamide Kidney cortex was not as deeply depressed by 50 μM iodipamide however as one would expect for system (1) with k_1 below 1 μM It seems therefore that even the hippurate insensitive iodipamide system *B* is composite One part has high affinity for iodipamide despite the high concentration of hippurate Another part has less affinity for iodipamide and/or more affinity for hippurate and therefore less

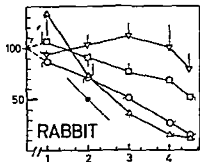


Fig. 6

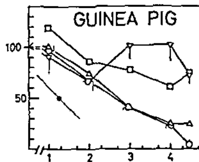


Fig. 7

Fig. 6. Hippurate depression of iodipamide uptake in different rabbit tissues. 7 rabbits each contributed 2 slices of kidney cortex, 2 slices of liver, 1 piece of anterior uvea and 1 piece of choroid plexus at each concentration. The control bottle had 2 pieces of plexus. Average incubation time kidney, liver 23, plexus 20, uvea 21 min; iodipamide concentration 2.4 μM. Abscissae: log μM hippurate; ordinates: tissue/medium ratio in per cent of controls. Circles kidney, squares plexus, Δ eye, ∇ liver. Bars: 1 S.E. (in contrast to previous figures). Control tissue/medium ratios for kidney 19.6±1.7, plexus 10.15±0.61, anterior uvea 5.01±0.90 and liver 1.89±0.29 (n = 7).
All figures corrected for unspecific uptake.
The isolated line shows the single system slope.

Fig. 7. Hippurate depression of iodipamide uptake in different guinea pig tissues. 30 guinea pigs, the protocol is explained in the text. Incubation time 20—22 min. The values shown for liver and kidney are means±S.E., for eye and plexus medians. Iodipamide concentration 2.5 μM, 0.04 μM Rose Bengal was also present throughout. Control tissue/medium ratios for kidney 8.15±0.26, for plexus 12.57±0.66, anterior uvea 2.28±0.09, liver 2.72±0.13 (n = 30). In all other respects the legend for Fig. 6 holds.

apparent affinity for iodipamide in the presence of much hippurate. The same conclusion can be drawn from Fig. 5. Here the hippurate sensitivity was tested in the presence of 12.5 μM iodipamide. This concentration, as Table I shows, must very largely saturate the most sensitive iodipamide system (1). Nonetheless a hippurate insensitive uptake remains in kidney as well as plexus.

Iodipamide uptake into rabbit kidney cortex thus seems to take place by at least three systems:

the hippurate sensitive, in common with iodohippurate (and presumably PAH);

a hippurate insensitive one, with very high affinity for iodipamide and very little for hippurate;

a hippurate insensitive one, with less affinity for iodipamide (or somewhat more for hippurate).

The distinction between the latter two systems has not yet been fully worked out. For the present purposes we shall distinguish only between the hippurate sensitive and the hippurate insensitive transport of iodipamide.

II. Iodipamide accumulation in other rabbit tissues

Slices of rabbit liver accumulate but little iodipamide under our conditions. Specific tissue/medium ratios for liver, anterior uvea, choroid plexus and kidney cortex from the same 7 animals are given in the legend of Fig. 6. Liver accumulates 10 times

TABLE II Simultaneous uptake of iodipamide and o-iodohippurate in rabbit tissues. Tissue/medium ratios ± S.E. are given uncorrected for unspecific uptake. Figures in brackets are number of animals, the statistical unit. With few exceptions any one animal contributed 4 kidney slices, 2 pieces of anterior uvea and 1 piece of choroid plexus to each of no inhibitor and 30 mM hippurate. All these animals and a few more contributed 2 kidney slices and 1 piece of anterior uvea to 12.6 mM iodipamide. Data for choroid plexus 12.6 mM iodipamide are from other animals treated in exactly the same manner except that only some had labelled iodohippurate in the medium. Incubation time: kidney 20, plexus 20, uvea 20–30 min. 1.25 μM iodipamide and 0.6 μM o-iodohippurate in the medium.

		Iodohippurate	Iodipamide
Kidney cortex	No inhibitor	6.900 ± 0.410 (16)	15.680 ± 1.060 (16)
	30 mM hippurate	0.880 ± 0.014 (17)	3.200 ± 0.160 (17)
	12.6 mM iodipamide	*0.910 ± 0.024 (22)*	*0.643 ± 0.030 (22)*
Anterior uvea	No inhibitor	5.220 ± 0.550 (15)	8.480 ± 0.890 (16)
	30 mM hippurate	0.890 ± 0.014 (17)	1.449 ± 0.042 (18)
	12.6 mM iodipamide	0.890 ± 0.010 (22)	0.990 ± 0.020 (22)
Choroid plexus	No inhibitor	2.550 ± 0.200 (12)	13.860 ± 0.970 (12)
	30 mM hippurate	0.866 ± 0.020 (12)	5.790 ± 0.450 (12)
	12.6 mM iodipamide	0.870 ± 0.017 (12)	0.660 ± 0.032 (23)

less than kidney cortex. This undoubtedly is due to active transport out of the liver cells at the canalicular end—iodipamide appears in much higher concentrations in the bile than in the liver parenchyma (see Sperber and Sperber 1971).

The influence of hippurate on iodipamide uptake in the 4 tissues is shown in Fig. 6. In this group of experiments the specific activity of the labelled iodipamide available was unusually low and the chemical concentration used, 2.4 μM, more than half depresses the highly sensitive iodipamide uptake system (1) of the kidney. Since, as we now know, system (1) is at least partly very insensitive to hippurate, the degree of depression by hippurate seen in Fig. 6 must be exaggerated. Nonetheless the difference between the tissues is clearcut. Liver is very insensitive to hippurate, choroid plexus evidently contains a large hippurate insensitive fraction, while in the anterior uvea (ciliary processes) and the kidney the hippurate insensitive fraction remaining under these conditions is 10—15 per cent.

The hippurate insensitive iodipamide uptake left at 10 mM hippurate can be depressed by iodipamide. In the rabbit liver only two such experiments were made. 100 μM iodipamide reduced uptake to 45 and 41 percent while uptake in choroid plexus was simultaneously reduced to 25 and 28 percent. High concentrations of iodipamide depressed the tissue/medium ratio for rabbit liver to 0.59 ($n = 7$).

The high hippurate insensitive fraction of iodipamide uptake in rabbit choroid plexus has already been noted. In 58 controls from other experiments with 10 mM hippurate present the mean tissue/medium ratio was 7.9. The chemical concentrations of iodipamide present in all these experiments varied and therefore no standard error is given for the figure. In Table II the special characteristics of choroid plexus are evident. *It accumulates iodohippurate poorly and iodipamide avidly.* The latter uptake is largely resistant to hippurate, the former not.

TABLE III. Temperature sensitivity of iodipamide uptake in guinea pig tissues. Figures show tissue/medium ratios uncorrected for unspecific uptake. Incubation time 20 min. One lateral choroid plexus, 2 slices of kidney cortex and liver behind each figure. 1.3 μM iodipamide in medium.

	No hippurate		30 mM hippurate	
	37 °C	0 °C	37 °C	0 °C
kidney cortex	8.86	0.53	1.56	0.50
liver	2.47	0.64	2.06	0.44
choroid plexus	13.49	0.39	9.58	0.45

III. Iodipamide accumulation in guinea pig tissue

The guinea pig eye and choroid plexus are so small that one whole anterior uvea and the whole lateral ventricular plexus of one side had to be used in each incubation bottle. One bottle was used as control, the other had a certain concentration of hippurate or 12.6 mM iodipamide. In order to reduce inter-individual variation inbred animals would have been desirable but were not available. Instead male guinea pigs of the same strain from a good colony were used in the weight range 300—400 g. One anterior uvea, one lateral choroid plexus, two liver slices and two kidney slices from each animal were incubated in one bottle. Each day six animals were used with all different concentrations and the percentage uptake figures of 5 such sets were averaged. In each of the eye and plexus data there was one value which fell far outside the group. Therefore medians instead of means are plotted for these tissues. Fig. 7 shows the results. The picture is evidently very similar to that in the rabbit: choroid plexus and liver have a large fraction of hippurate-insensitive uptake, kidney and anterior uvea a smaller one.

Kinetic constants were obtained by computer fitting 2 Michaelis-Menten uptake systems to the data similarly as in Table I. The half-saturation concentrations of hippurate of the hippurate-sensitive fractions were in μM: kidney 113, plexus 75, eye 186. The differences are probably not significant. Choroid plexus has about 75 per cent of a hippurate-insensitive uptake system, eye and kidney about 30 per cent.

Table III finally shows that the hippurate-sensitive and the hippurate-insensitive systems both are inhibited by cold.

IV. Iodipamide uptake in monkey tissues

Kidney, liver and choroid plexus from 10 cynomolgus monkeys, one stumptail macaque and one vervet monkey were available at the termination of long term eye experiments. The tissues were not all tested according to the same protocol and all data relevant for the present question are not available for each animal. Table IV therefore gives the figures for the individual animals. The means serve only for a very rough orientation.

TABLE IV Uptake of iodipamide by tissues of freshly killed monkeys 2 slices of kidney cortex, 2 of liver and one piece of lateral choroid plexus behind each individual tissue/medium ratio Average incubation time 23 min plexus 20 min except 519 Iodipamide concentration in medium 1 3 μM except in 466 513 and 514

Species	No	Unspecific uptake			Specific no hippurate			Specific 10 mM hippurate			Comments
		kidney	plexus	liver	kidney	plexus	liver	kidney	plexus	liver	
Cyno	466	0 82	0 87	—	7 70	11 58	—	0 85	9 01	—	0 5 μM iodipamide
	513	—	—	—	6 73	—	—	0 68	—	—	3 μM iodipamide
	514	0 63	—	—	6 42	—	—	0 69	—	—	3 μM iodipamide
	518	0 61	0 62	0 82	6 97	6 57	2 93	0 74	4 99	2 46	
	519	0 51	0 94	0 48	7 39	10 53	4 14	—	—	—	plexus incub 20 min av kidney 27 liver 40
	520	0 60	0 71	1 13	—	—	—	0 67	4 54	0 65	
	521	0 70	0 47	0 60	7 49	4 69	1 61	—	—	—	
	522	0 69	1 17	0 80	8 19	9 82	2 61	—	—	—	
	523	0 75	0 96	1 19	—	—	—	0 51	2 71	1 33	
	525	0 67	0 72	0 87	6 71	11 20	1 57	—	—	—	
Mean		0 66	0 81	0 84	7 20	9 07	2 57	0 69	5 31	1 48	
Stumptail	524	0 70	0 63	—	—	—	—	0 67	3 76	—	
Vervet	528	0 75	0 44	0 99	6 08	10 29	1 56	0 69	6 64	1 77	

TABLE V Uptake of iodipamide by tissues of young monkeys several hours post mortem One piece of choroid plexus and 1–2 pieces of ciliary body behind each individual tissue/medium ratio Average incubation time plexus 20 eye 22 min Experiments linked by brackets used tissue from the same head

	Unspecific uptake		Specific no hippurate		Specific 10 mM hippurate		Specific 30 mM hippurate		Start of incubation hrs after death	Comments
	plexus	eye	plexus	eye	plexus	eye	plexus	eye		
No 526	0 83	0 63	3 90	3.20	—	—	—	—	2	526–535 1 3 μM iodipamide
527	0 84	0 74	—	—	1 64	0 16	—	—	4	
532 }	0 81	0 69	6.52	1 77	1 04	0 03	2 94	0 15	2	averages
533 }	0 81	0 69	2 92	2 33	1 77	0 17	0 93	0 25	3	last hour at 37 C in Petri dish
534 }	0 81	0 69	3 96	5 34	2 90	0 50	2 74	0 08	4 5	last hour at 37 C in Petri dish
535 }	0 81	0 69	2 14	2 87	1 69	0 24	2 25	0 04	5 5	last hour at 37 C in Petri dish
540 }	0 81	0 69	11 37	1 93	6 91	0 21	2 98	0 10	2	540–543 2 4 μM iodipamide
541 }	0 81	0 69	9 53	1 28	6.29	0 22	3 48	0 08	3	Last hour at 0 C in Petri dish
542 }	0 81	0 69	5 07	2 00	1 98	0 00	2 27	−0 04	4	
543 }	0 81	0 69	4 37	1 72	5 99	−0 02	1 75	−0 12	5	Last hour at 0 C in Petri dish
Mean			5 53	2 49	3 36	0 17	2 42	0 068		

TABLE VI Uptake of iodipamide by cat tissue 2 slices of kidney cortex or liver 1–2 pieces of choroid plexus and ciliary body behind each individual tissue/medium ratio Average incubation time plexus 20 kidney 23 eye 23 liver 24 min Iodiamide concentration 0 5 μM in 481–496 2 5 μM in 602–618

No	Age	Specific no hippurate				Specific 10 mM hippurate				Specific 30 mM hippurate				
		kidney	plexus	liver	eye	kidney	plexus	liver	eye	kidney	plexus	liver	eye	
481	adult	3 69	6 10	—	—	2 38	3 95	—	—	—	—	—	—	
482		3 13	6 18	—	—	2 00	5 26	—	—	—	—	—	—	
493		3 25	9 19	—	0 79	2 44	3 81	—	0 38	—	—	—	—	
496		4 13	4 06	—	1 65	3 51	4 95	—	1 15	—	—	—	—	
603		1 96	2 48	2 36	0 58	1 59	2 73	2 07	0 42	0 90	1 52	1 05	0 31	
Mean		3 23	5 60		1 01	2 38	4 14		0 65					
618	9 hrs	0 16	1 85			0 10	1 62			0 07	1 61			litter of 3
602	*2 weeks*	*0 48*	*3 94*			*0 30*	*2 73*			*0 25*	*1 68*			*litter of 4*

The table shows that in 3 monkey species there is a large hippurate insensitive fraction of iodipamide uptake in the choroid plexus and a small one in the kidney cortex. Whether there is a hippurate *sensitive* uptake in the liver is uncertain. Uptake in the liver is distinctly smaller than in kidney or plexus

In order to study the system in the eye the heads of very young cynomolgus monkeys killed for vaccine production were put into iced saline shortly after death and brought to the laboratory where they arrived about 2 hours later The choroid plexus and the eyes were utilized It proved difficult in these very young animals to remove the lens by purely mechanical means without tearing off some ciliary epithelium Therefore in experiments 540—43 a few drops of chymotrypsin were injected into the zonular region from the vitreous side the lens partly frozen with a chilled probe and then removed with a minimum of trauma to the ciliary processes The surface of the ciliary epithelium was quickly washed off the iris dissected from the ciliary body and sectors of ciliary body taken for incubation

Table V shows the results Evidently considerable active iodipamide uptake *remained in the tissues despite the long storage of the heads at 0° C Compared* with Table IV where the tissues were taken out immediately after death uptake seems to be about half Choroid plexus evidently contained a considerable hippurate insensitive fraction while this fraction was much less in the ciliary processes The chymotrypsin procedure seems to be better than simple mechanical removal of the lens Allowing the preparation a recovery period at 37 C in shallow medium exposed to air caused more deterioration in uptake than keeping the preparation at 0 C A very rough estimate of the fraction of plexus uptake left at 10 mM hippurate gives 60 per cent for Table IV as well as for Table V It seems therefore that the hippurate sensitive uptake mechanisms is about as resistant to asphyxia as the insensitive one

TABLE VII Uptake of iodipamide by dog tissues 2–4 pieces of kidney cortex plexus and ciliary body behind each tissue/medium ratio in the uninhibited 2 pieces of kidney and eye and 1 of plexus in the inhibited columns 7 5 µM iodipamide throughout Incubation time plexus 20 kidney and eye 23 min Unspecific uptake kidney 0 86 plexus 0 63 eye 0 77

No	Specific no inhibitor			Specific 3 mM probenecid			Specific hippurate	
	kidney	plexus	eye	kidney	plexus	eye	10 mM plexus	30 mM plexus
576	0 93	3 07	0 42	0 40	0 47	0 27	—	—
577	0 79	3 22	0 25	0 56	0 46	0 03	—	—
581	—	7 62	—	—	—	—	3.26	2 40

I *Iodipamide uptake in tissues from the cat dog and chicken*

Two litters of very young *cats* and a small number of young adults were investigated. Table VI summarizes the results There is remarkably little hippurate *sensitive* uptake in the cat the whole level is low and a relatively much larger uptake remains in the presence of hippurate than in the monkey, rabbit or guinea pig Plexus and liver are quite similar and very insensitive to hippurate

In the new born kitten there is very little uptake in the kidney but appreciable and highly hippurate resistant uptake in the choroid plexus At two weeks of age the kidney still accumulates but little while the choroid plexus is not much less active than in the adult animal

A few experiments were done with tissue from 3 adult *dogs* The uptake by kidney cortex and ciliary body was decidedly less than in the other species tested while uptake by choroid plexus was appreciable (Table VII) The uptake by plexus was inhibitable by probenecid but only poorly so by hippurate (only the plexus was tested)

The poor uptake in kidney slices agrees with findings by Berndt and Mudge (1968) who found no evidence for secretion of iodipamide by dog kidney and very little uptake by slices of kidney cortex which accumulated PAH normally

As in the other species the uptake by ciliary processes is similar to that in the kidney while plexus differs in having much more of the hippurate insensitive iodipamide accumulating system

A *chicken* kidney had a maximum T/M of 5 4 of which 34 per cent remained in the presence of 2 mM hippurate In another chicken kidney specific T/M was 0 82 in the presence of 10 mM hippurate which is 15 per cent of full uptake a figure similar to that found in several mammals This uptake was reduced to less than half by 0 7 mM probenecid

Probenecid

Probenecid inhibits the hippurate sensitive as well as the hippurate insensitive system In the rabbit kidney cortex it has a higher affinity to the iodohippurate accumulating hippurate sensitive system than to the hippurate insensitive one Fig

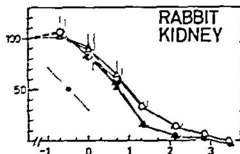


Fig 8

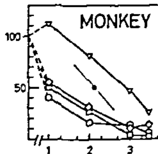


Fig 9

Fig 8 Probenecid depression of the uptake of o-iodohippurate and iodipamide in rabbit kidney cortex. 10 animals 2 control bottles 1 at all other concentrations 2 slices per bottl Abscissae log probenecid concentration in μM ordinates tissue/medium ratios in per cent of controls Open symbols iodipamide filled symbols o-iodohippurate Circles 20 min incubation triangles 30 min. Bars 2 S E Control tissue/medium ratios for iodipamide 20 min 19 9 ± 1 6 30 min 24 8 ± 1.8 for o-iodohippurate 20 min 8 96 ± 0 89 30 min 9 52 ± 0 78 n = 20)
Concentration of labelled iodipamide 0 1 μM of o-iodohippurate 0 14 μM.
The isolated line shows the single-system slope

Fig 9 Probenecid depression of iodipamide uptake in monkey tissues Choroid plexus from 1 stumptail macaque run in the presence of 10 mM hippurate kidney liver and plexus from 1 cynomolgus macaque run without hippurate One piece of plexus 2 slices of liver and kidney in each bottle only one control bottle Incubation time plexus 20 min, liver kidney 23 (av) Abscissae log μM probenecid Circles kidney squares plexus triangles liver diamonds plexus with 10 mM hippurate
Control tissue medium ratios kidney 6 7 liver 1 6 plexus without hippurate 11 plexus with hippurate 3 8 Concentration of labelled iodipamide 1.3 μM
In other respects the legend for Fig 8 holds

TABLE VIII Kinetic constants derived from the data of Fig 8 The explanation of Q and U is as for Table I The k values are half-saturation concentrations of probenecid in μM

Kind of average	Incubation time mins	Iodipamide uptake					
		Q_1	k_1	Q_2	k_2	U	
mean	20	60	2 4	40	54	3 4	
mean	30	82	6 5	18	160	1 4	
median	20	5	1.5	48	39	4.2	
median	30	86	6 3	14	250	1 8	
mean	20 – 30	0	4.2	50	79	2 4	
median	20 – 30	4	1 8	53	32	2 8	
mean	20	49	0 50	51	29	1 4	point with zero probenecid discarded
mean	30	9	5 7	21	120	1 3	point with zero probenecid discarded

8 shows an experiment with simultaneous uptake of iodohippurate and iodipamide *It is evident that the uptake of iodohippurate reaches virtually total suppression before the uptake of iodipamide* Computer analysis into two uptake systems (as in Table I) of the curve for iodohippurate suggests one dominant system which is

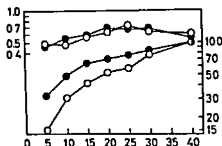


Fig 10 Rabbit kidney cortex Time course of un inhibited iodipamide and o-iodohippurate uptake and of degree of partial inhibition by 10 μM probenecid 2 separate experiments each animal contributing one inhibited and one uninhibited slice for each incubation time Abscissae incubation time mins The lower pair of curves reads against the right hand ordinate which show tissue/medium ratios in uninhibited slices with the 40 min value set at 100 percent The upper pair of curves reads against the lefthand ordinate and shows the quotients between inhibited and uninhibited tissue/medium ratios Open symbols iodipamide filled symbols o-iodohippurate

Uninhibited tissue/medium ratio at 40 min iodipamide 23 7 o-iodohippurate 9 8 (mean of 2 values)

Concentration of labelled iodipamide 0 5 μM of o-iodohippurate 1 6 μM All figures corrected for unspecific uptake

half saturated by 4—6 μM probenecid The iodipamide uptake curve analysed in the same manner indicates two uptake systems (Table VIII) One system Q_1k_1 is half saturated by 0 5—6 5 μM probenecid thus in the same concentration range as the iodohippurate system The other Q k needs much more probenecid (29—156 μM) The size of the two systems is somewhat uncertain but they are both appreciable Possibly the probenecid sensitive system (1) is somewhat larger A comparison with Table I shows that this system (1) of Table VIII can very well be the same as system (2) of Table I the less iodipamide but more hippurate sensitive

There is too little material for a quantitative analysis of the systems in plexus and eye but in these tissues too iodipamide uptake keeps better than uptake of iodohippurate when the probenecid concentration goes up This is especially marked for plexus where the hippurate insensitive system is large

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As mentioned in the Methods section the degree of inhibition by 10 μM probenecid decreased somewhat with time Fig 10 shows the available data (2 expts) Also in Fig 8 2 and 3 there are differences between the 20 and 30 min averages which may or may not be significant Time dependence of inhibition has not been taken into account in the mathematics behind the analysis into 2 uptake systems used in the present paper It may be an important clue to mechanisms (see Discussion)

Discussion

There are previous reports indicating a composite nature of the organic acid transporting system in the eye and in the kidney Cunha Vaz and Maurice (1967) who studied the transport of fluorescein out of the rabbit vitreous cavity found evidence for a two part system one of which was blocked only by very high

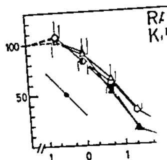


Fig 8

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50
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Fig 9

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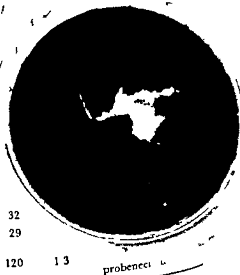


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probeneci

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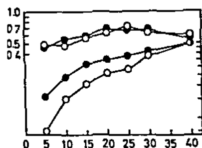


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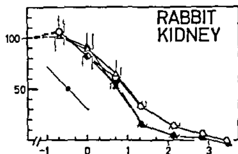


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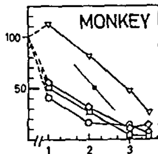


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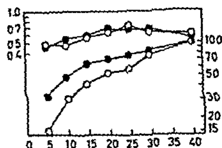


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concentrations of inhibitors May and Weiner (1971) studying the renal excretion of *meta* hydroxybenzoate in the *Cebus* monkey likewise found evidence for more than one secretory system for organic anions

While it seems unquestionable that iodipamide uptake takes place by a composite system which partly differs from the hippurate uptake system the kinetic constants derived from our data are not beyond doubt Slices are not ideally thin and diffusion barriers may affect the results The incubation time is a compromise between the need for appreciable uptake and the desire to stay on a reasonably steep part of the time uptake curve The assumption of only two uptake systems is especially doubtful First of all there could be more than 2 uptake systems for iodipamide, some evidence has been presented But since the liver cell almost certainly has an active outwards directed system for iodipamide it is not impossible that some vestiges of a similar system exist in other tissues Competition could then occur for uptake as well as for outwards directed transport The fact that hippurate in low concentrations increased the tissue/medium ratio for iodipamide a little (Fig 3) could be due to this With Rose Bengal a halogenated fluorescein which very effectively inhibits iodipamide uptake, such phenomena were very evident in a small series of guinea pig experiments (unpublished) Hippurate at 30 mM did not depress but increased uptake by 9, 21 23 and 42 per cent in kidney cortex liver plexus and eye respectively while iodipamide at 12 6 mM more than doubled the specific tissue/medium ratio in kidney cortex slices It is clear that at least for ose Bengal the simple model of 1 or 2 straightforward uptake system is not fficient and such a more complex situation also for iodipamide cannot be ruled ut with certainty It would tend to exaggerate the size of the hippurate insensitive system On the other hand the figures given for the size of the system in Table II—VII may be misleadingly small since as Fig 3 6 and 7 indicate it is possible that hippurate insensitivity is not complete It could be that a superhippurate exists that has no affinity at all to part of the iodipamide uptake systems but there is no *a priori* reason why hippurate should have this ideal selectivity

For the iodohippurate transporting system there is no doubt about the direction in which secretion takes place in the body For the hippurate insensitive iodipamide system this matter is not settled The rabbit tubules secrete iodipamide into the urine (Berndt and Mudge 1968) but the secretion observed could be by the hippurate sensitive part The direction of secretion in the liver is evident but for the kidney eye and plexus the matter will have to be investigated and until this is settled speculation about physiological function is premature

The fact that probenecid inhibits the system albeit at higher concentrations than the ordinary hippurate system underlines the limited selectivity of this drug and may explain why the new system has not been discovered previously

Levi Arias and collaborators have described two cytoplasmic anion binding proteins Y and Z located mainly in the liver (Levy Gatmaitan and Arias 1969) Protein Y has recently been christened Ligandin (See Litwack Ketterer and Arias 1971 this letter also gives recent references) Several of the substances reported to

be bound by these proteins are efficient depressors of the hippurate-insensitive iodipamide system studied here (Bárány unpublished). At present it seems therefore not completely impossible that, as suggested by Levy *et al.* (1969), part of the phenomena studied here are due to intracellular interactions: competition for intracellular binding sites. As pointed out (Bárány 1971) the mathematics would become very similar but the time relations of the competition would differ. Inhibition would as a rule increase with time as saturation of intracellular binding sites progresses. The opposite was in fact observed (Fig. 10). Nonetheless the matter evidently needs further investigation by other methods.

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The Sodium Transport Pool in Frog Skin under Open and Short circuit Conditions

By

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Abstract

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The sodium pool of frog skin was studied by a method of continuous direct analysis under open and short-circuit conditions No change in the transport pool on short circuiting was found The results are discussed on the basis of proposed models for the sodium transport in frog skin

efoed Johnsen and Ussing (1958) presented a model of the frog skin consisting of a outer passively but selectively permeable barrier and an inner Na barrier and in etween a homogeneous Na transport compartment whose Na content is generally called the Na transport pool The active transport of Na was considered to be localized to the inner barrier Since the time of this report the interest in studies of the transport system in frog skin has been focused increasingly on the Na pool see Herrera (1970) and Cereijido and Rotunno (1970) for review

In studies of the Na pool in frog skin some authors use an open circuit procedure (*i e* the two sides of the frog skin are not in external electrical communication with one another) while others use a short circuit method (*i e* the potential difference (PD) across the skin is decreased towards zero by means of an external source of current) according to the method of Ussing and Zerahn (1951)

No comprehensive studies have been made of the effect of short circuiting on the Na pool and comparison between the results of studies on skin in open and short circuit conditions therefore offer difficulties

The aim of the present investigation was to study the Na pool in frog skin under open and short-circuit conditions

Methods presented hitherto for studies of the Na pool either have involved destruction of tissues or have been very time consuming and thus the possibilities of studying the Na pool under different experimental conditions (*e g* hyper and hypopolarization alteration of the ionic environment) but on the same experimental

object have been limited A method has therefore been developed allowing con tinuous direct detection of the content of a radioactive tracer substance in a mem brane system under variable, reproducible conditions (Lyttkens and Sjostrand 1970) With this method it is also possible to study the accumulation and washout of radioactive tracer in the membrane system with good time resolution

Methods

Biological object

The experiments were performed during the months September to December on abdominal skin from frogs Rana temporaria of both sexes The animals were kept in circulating tap water at a temperature of 8 C

Solutions

The Ringer s solution used was the same as used by MacRobbie and Ussing (1961) and had the following composition 1 08 mM CaCl 2 4 mM KCl 111 1 mM NaCl and 2 4 mM NaHCO and pH 8 0

Radioactive Ringer s solution with the same composition as above was prepared with Na from the Radiochemical Centre Amersham England delivered in 0 9 % NaCl

Perfusion-superfusion system

A water silicone oil system has been developed and its properties have been studied in detail (Lyttkens and Sjostrand 1970) see Fig 1—3 Briefly the system allows a continuous study of the content of a radioactive tracer substance within a membrane (biological or artificial) under variable reproducible conditions This is rendered possible by superfusion of silicone oil over the surfaces of the membrane whereby as shown in the previous study this becomes coated with thin aqueous films (in the present case films of Ringer s solution) the thickness of which has been found to be 4 µm±2 µm on a millipore filter membrane By this means detected radioactivity will emanate mainly from the membrane system while only a minor proportion will emanate from the surface films An optimal superfusion pressure (0 4—0 5 kp/cm) and a perfusion rate with an ensured washing effect on the membrane surfaces (0 1 ml/min) were used throughout as described in the previous paper The pressure difference between the two sides of the membrane chamber could be checked and adjusted so as to obtain identical pressures on the two sides The stability of the short circuit current during the experiments also excluded minor non measurable pressure difference across the frog skin (Nutbourne 1968) The pressure in the membrane chamber in relation to the surrounding atmospheric pressure was adjusted to 0±1 mm Hg In relation to the perfusion super fusion system presented previously the following modifications were made

The radioactive solution was introduced by means of a separate injection device via a narrow cannula (inner diameter 0 1 mm outer diameter 0 2 mm) to the upper margin of the exposed frog skin surface (see Fig 3) The radioactive solution was perfused at a rate of 0 09 ml/min and at the same time an inactive solution was perfused via the filter paper (see Fig 3) at a rate of 0 01 ml/min In this way radioactive tracer was prevented from diffusing into the filter paper (see Detection system for direct analysis below) Experiments in which a coloured solution was injected via the cannula showed that the silicone oil superfusion distributed the injected solution homogeneously over the surface of the frog skin

The silicone oil was saturated with oxygen by bubbling During the experiments the silicone oil was continuously superfused over the surface films of Ringer s solution In this way oxygen in the silicone oil will exchange with the gases in the Ringer s solution which will hereby be oxygenated Measurements on perfusate solution (see Sampling system for flux measurements below) with a Po electrode (type E 5046 Radiometer Copenhagen Denmark) rendered Po values equal to atmospheric pressure The resting potential between 20 and 60 mV and the short circuit current were stable throughout the experiments indicating adequate oxygenation of the Ringer s solution

In this study the system was controlled at 16 ±0 5 C

Detection system for direct analysis

In this system a semiconductor detector was used and recording of the amplified and discriminated signal took place in a multichannel analyser system at multiscaler mode with an analysis time of 15 sec for each channel In relation to the equipment described previously (Lyttkens and Sjostrand 1968 1970) the detection system was modified as follows

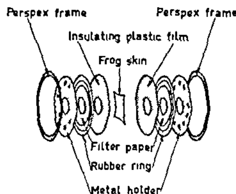


Fig 1

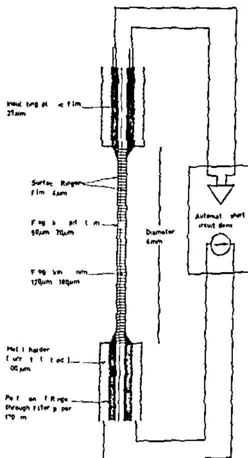


Fig 2

Fig 1 Diagram showing the mounting of the frog skin

Fig 2 Diagram showing the different dimensions in mounting of the frog skin and the automatic short circuit device

A new detector (SJ 2420 RCA Victor Co Ltd Montreal Canada depletion depth 0.? mm active area 5 mm²) was placed on the side opposite to the radioactive perfusion (see Fig 3)

As the detector was somewhat sensitive to γ radiation (with ²⁴Na ~ ... efficiency) high initial counting rate emanating from radioactivity (in this study ²⁴Na) in the afferent channels of the membrane chamber was obtained as well as radioactivity in the filter paper (total volume about 1 ml) With the beta and gamma radiation source used this gave an unfavourable signal/ background ratio and therefore the radioactive Ringer's solution was injected through a cannula to the upper margin of the exposed membrane surface (see Perfusion superfusion system above and Fig 3) By this means the high radioactivity from the afferent system used in the previous study was eliminated

Mounting and short-circuiting of the frog skin

Mounting

A piece of frog skin measuring 1 ... was mounted as illustrated in Fig 1–3 The mounting procedure took about 15 min in all On mounting a circular area of the frog skin with a diameter of 4 mm was exposed

Fig 3 Schematic illustration (vertical section) of the superfusion perfusion detection and perfusate sampling systems and the automatic short circuit-device For details see text

Short-circuiting procedure

Ussing and Zerahn (1951) decreased the potential across the frog skin towards zero by means of an external source of current and in their experimental arrangement the skin was homogeneously short-circuited As the short circuit current in this study was applied through two circular electrodes (platinized phosphor bronze Fig 1–3) and as the skin was covered by thin (4 μm) surface films of Ringer's solution it was not possible to achieve homogeneous short circuiting of the skin as will be discussed further below The current through the circular electrodes was applied by means of an automatic short-circuit device This device adjusted the current (according to the negative feed back principle) until there was no (0±0 5 mV) potential difference between the two electrodes that measured the potential across the frog skin (Fig 2) The electrodes for potential measurement were of a hybrid type (porous silver/silver chloride/platinum black type GWH? In Vivo Metric Systems Redwood Valley Calif USA) and as they measured the potential across the peripheral part of the exposed frog skin (Fig 2) only this part of the skin was short-circuited During short circuiting the central parts of the skin had a potential difference (inside positive) The applied by the short circuit device was recorded continuously throughout the experiments

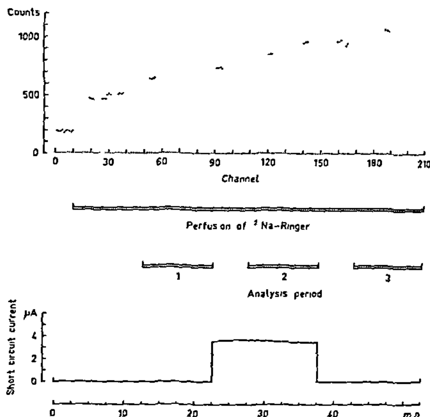


Fig 4 The sequence of analysis in direct detection of the Na pool

Measu ement of background rad oactiv ty in direct detection

This measurement was performed identically to that in the b ological experiments with the exception that water repellant plastic foil replaced the frog skin

Owing to the construction of the chamber and of the perfusion superfusion system part of the Ringer s solution that was washed away from the membrane surfaces accumulated as drops in the chamber (as indicated in Fig 3) As the detector had an efficiency of ~5 for γ radiation the accumulating drops on the rad oactive side were recorded as an increase in the background acti ity (see Results below and Fig 6) In experiments lasting up to 4–5 h this accumulation reached a saturation level which oscillated owing to random washing away of radioactive drops

Samplin system fo flux measu ement

To allow flux measurements the system was supplied with a sampling device As shown in Fig 3 those drops of perfusate that were washed away by the sil cone oil superfusion were allowed to equilibrate with a solution consisting of 100 ml of 0 9 NaCl Samples of this soluti n were then measured by analysis of the Čerenkov radiation (Sjostrand *et al* 1969) in a Liquid Scint llator System (Beckman LS 250)

Procedure

Analysis sequence in di ect dete tion of the Na pool

Fro skin

The sequence of analysis in direct detection of the Na pool is illustrated in Fig 4 During 2 5 min of perfusion of inactive Ringer's solution (0 10 ml/m n) over both surfaces of the frog skin the initial radioactivity was recorded (radioactivity that had been previously adsorbed in the chamber radio-

activity from the afferent system *etc*) The perfusion of radioactive Ringer s solution over the epithelial surface of the frog skin (0 09 ml/min) was then started and at the same time the inactive perfusion of this side was reduced to 0 01 ml/min This type of perfusion was th n maintained throughout the experiment During the first 20 min the skin was kept in open-circuit of which 10 min comprised equilibration time and 10 min analysis time (analysis period 1) In the following 15 min the skin was kept in short-circuit—5 min equilibration and 10 min analysis (analysis period 2) For the last 15 min the skin was kept in open-circuit—5 min equilibration and 10 min analysis (analysis period 3)

Measurement of background radioactivity in direct detection

The sequence of analysis in the background studies consisted of 2 5 min of inactive perfusion over both sides and 50 min of radioactive perfusion over one side (the same perfusion rates as above) Analysis periods 1 2 and 3 comprised the channels 51—90 111—150 and 171—210 *i e* analogous to the corresponding analysis periods in the frog skin studies

Sepa ate direct analysis of the si e of the Na pool

Control studies of the size of the Na pool were made by means of separate direct measurements of the Na pool The measurements were performed on frog skin in which [24]Na in Ringer s solution from the epithelial side was allowed to equilibrate for 30 min After the skin had been dried a piece 4 mm in diameter was punched out This piece of skin was mounted on plastic foil so as to cover the hole in the metal holder exactly The measurement was then performed during sil cone oil superfusion but without simultaneous perfusion with Ringer s solution By this means any risk of washing out of Na of the epithelium was el minated and therefore the radioacti ity obtained (after background subtract on) in this analysis situation emanated only from the Na pool

Analysis sequence on flux measurement

In flux studies the epithelial surface of the frog skin was perfused with active Ringer s solution and the corium side of the skin with inactive Ringer s solution (the same perfusion rates as above) The analysis s quence comprised 70 min open-circuit 70 m n short circuit and 70 min open-circuit The perfusate from the inside of the frop skin was equilibrated with 100 ml of 0 9 NaCl (see Methods above and Fig 3) This solution was changed every 10 m n and two 10 ml samples were analysed in a liquid sc ntillation counter by Čerenkov analysis

Calculations

Correction for decay of [24]Na was made in all experiments

As the samples from the flux studies mentioned above were colourless and as the Na was measured by Čerenkov analysis correction for quenching was unnecessary (*cf* Lyttkens and Sjostrand 1971)

The circular short c rcuit electrodes used in this system in the periphery of the exposed frog skin gave a maximal dens ty of current in the peripheral parts of the skin which were short-circuited (PD = 0 mV) The current density then decreased gradually towards the central parts of the skin where the ins de had a positive potential (see Fig 2) With homogeneous short ci cuiting of frog skin the short circuit current corresp nds to the transepithelial net flux of Na (Ussing and Zerahn 1951) Since the net flux of Na consists to a large part of influx of Na and to only a minor part of Na efflux the short-circuit current is often used as a measure of Na influx If the exposed frog skin d ring short ci cuiting is imagined to be divided schematically into 2 parts one part (A) which becom s homogeneously short-circuited and a second part (B) which does not become short circuit d at all the following equations are obtained for the influx from the ent re exposed frog skin surface (A+B) in open-circuit (Na_{oc}) and short circuit (Na_{sc}) and for the short-circuit current (I_{sc})

$$Na_{oc} = k_1A + k B \quad (1)$$

$$Na \quad = k_2A + k B \quad (2)$$

$$I_{sc} \quad = k_3A \quad (3)$$

The constants k and k des gnate factors by which parts A and B are to be multiplied to obtain the influx of Na under open-c rcuit and short-circuit condition respecti ely Development of the above three equations gives

$$\frac{A}{A+B} = \frac{Na_{oc} - Na_{sc} + I_{sc}}{Na_{oc}} \quad (4)$$

From equat on (4) and the val ies obtained for Na_{oc} Na_{sc} and I the area of A can be calculated in relat on to the whole exposed frog skin (A+B)

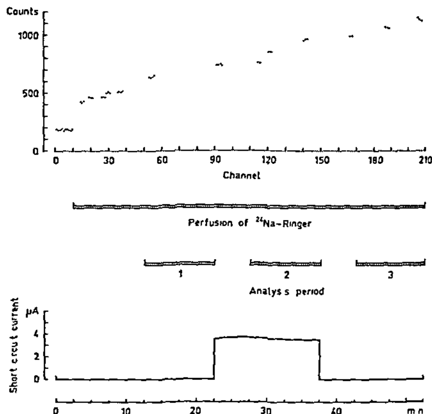


Fig 4 The sequence of analysis in direct detection of the Na pool

Measurement of background radioactivity in direct detection

This measurement was performed identically to that in the biological experiments with the exception that water repellant plastic foil replaced the frog skin

Owing to the construction of the chamber and of the perfusion superfusion system part of the Ringer s solution that was washed away from the membrane surfaces accumulated as drops in the chamber (as indicated in Fig 3) As the detector had an efficiency of ~5 for γ radiation the accumulating drops on the radioactive side were recorded as an increase in the background activity (see Results below and Fig 6) In experiments lasting up to 4 – 5 h this accumulation reached a saturation level which oscillated owing to random washing away of radioactive drops

Sampling system for flux measurement

To allow flux measurements the system was supplied with a sampling device As shown in Fig 3 those drops of perfusate that were washed away by the silicone oil superfusion were allowed to equilibrate with a solution consisting of 100 ml of 0 9 % NaCl Samples of this solution were then measured by analysis of the Čerenkov radiation (Sjostrand *et al* 1969) in a Liquid Scintillator System (Beckman LS 250

Procedure

Analysis sequence in direct detection of the Na pool

Frog skin

The sequence of analysis in direct detection of the Na pool is illustrated in Fig 4 During 25 min of perfusion of inactive Ringer s solution (0 10 ml/min) over both surfaces of the frog skin the initial radioactivity was recorded (radioactivity that had been previously adsorbed in the chamber radio-

TABLE I Values for influx of Na in open-circuit (Na_{oc}) and short-circuit (Na_{sc}) conditions and for the short circuit current (I_{sc}) The imagined homogeneously short-circuited part of the frog skin in percent of the total exposed frog skin calculated as described in the text is given

Frog skin number	Na influx		Short-circuit current mEq/cm² h I	Calculated percentage of exposed skin being short circuited
	Open-circuit mEq cm h¹ Na_{oc}	Short-circuit mEq/cm² h Na_{sc}		
1	0 5	0 9	0 6	40
2	0 4	0 9	0 6	25
3	0 6	0 8	0 5	50
4	1.2	1.8	1 0	33
5	2.3	3.2	1 6	30

The Na pool on short-circuiting

The situation in analysis of the Na pool is illustrated in Fig 6 which shows 2 consecutive expts a frog skin experiment and a background experiment the analysis sequences of which are given under Methods On the same occasions an experiment with 100 % loading of a millipore filter with ^{24}Na Ringer s solution was performed analogously to the study of Lyttkens and Sjostrand (1970 Fig 8 and 9) The radioactivity in the millipore filter membrane (155±5 μm thick containing 80 volumes % ^{24}Na Ringer s solution) and 2 surface films (each of 4 μm) was 1875 counts per channel (15 sec) From these values the radioactivity in the surface films were calculated to be 61 and 53 counts per channel respectively The volume of the surface film was 5 04 10^{-4} ml and as the Ringer s solution contained 113 5 mM Na 5 72 10^{-2} μmol Na was found in the surface film The radioactivity in the Na pool of the frog skin and the surface film with ^{24}Na (*i e* the surface film of the epithelial side) was on the average 219 counts per channel (the difference between the two curves in Fig 6) This mean value was calculated from all three analysis period (see Methods) since there was no change in the Na pool on short-circuiting as reported below The radioactivity from the Na pool amounted consequently to 166 counts per channel corresponding to a Na pool of 0 14 μmol/cm² which is in agreement with previous findings (Hoshiko and Ussing 1960 Andersen and Zerahn 1963 Curran Herrera and Flanigan 1963 Cereijido *et al* 1964)

To be able to compare experiments performed on different occasions a relative calculation was introduced as described above (see Methods) The results are given in Fig 5 The left side of the figure shows the situation in background analysis where the relative difference between the total number of counts in analysis periods 2 and 1 was 0 545±0 017 (n = 4) The right side of the figure shows the situation in frog skin analysis The corresponding difference here was 0 545±0 026 (n = 7) In both cases the variation was calculated as ±S D

As the radioactivity from the Na pool was determined in those experiments shown in Fig 6 (see above) the radioactivity from the sodium pool during analysis period 2 could be compared with the difference between the total number of counts during

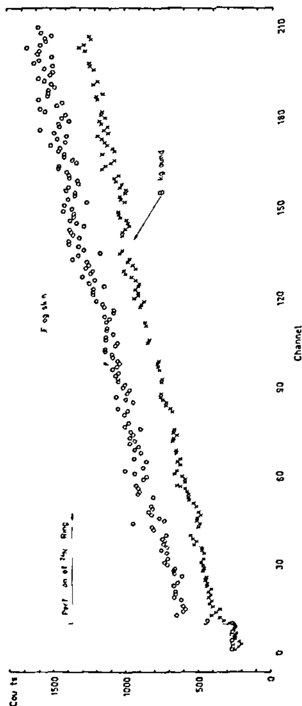


Fig 6 Results of analyses in two consecutive experiments — background and frog skin analysis

analysis periods 2 and 1 A corresponding comparison with the difference between the total number of counts during analysis periods 2 and 1 was made in control experiments ($n = 3$) where the Na pool was determined separately (see Methods) In the above experiments the Na pool comprised 50—70 % of the difference between the total number of counts during analysis periods 2 and 1 (on the relative scale 50—70 % of 0 545 *cf* Fig 5)

With the equilibration times used here (10 min before analysis period 1 and 5 min before analysis periods 2 and 3) the frog skin can be assumed to be in isotopic equilibrium during the respective analysis periods (Hoshiko and Ussing 1960 Andersen and Zerahn 1963) The Na pool can thus be assumed to have the same Na content during analysis periods 1 and 3 If the Na pool had altered during short-circuiting the relative difference between the total number of counts during analysis periods 2 and 1 for the frog skin experiments would differ from the corresponding difference for the background experiments Since these differences were identical (on the relative scale 0 545 see Fig 5) no change in the Na pool on short circuiting was found As the pool corresponds to 50—70 % of the difference between the total number of counts in analysis periods 2 and 1 and as that schematic part of the frog skin that under short circuit conditions becomes homogeneously short circuited corresponds to 36 % of the total exposed frog skin surface this part (A) in the relative scale amounted to 0 098—0 137

Discussion

The transepithelial net flux of Na increases in short circuit conditions (Ussing and Zerahn 1951) On the basis of the frog skin model described in the introduction this finding might be explainable by several different mechanisms

a) *An increase of the Na flux from the outside solution through the outer barrier to the Na transport compartment* whereby the total available Na at pumping sites would increase resulting in an increased net flux — In support of such a mechanism is the fact that PD (measured intracellularly) across the outer barrier is altered from having been positive to becoming negative under short circuit conditions Cereijido and Curran (1965) found values for PD across the outer barrier in Rana pipiens of −66 mV in open circuit and −18 mV in short circuit

b) *Elimination of epithelial Na shunts* might lead to an increase of the net flux of Na without a simultaneous change of the transport rate of the active transport mechanism —The presence of shunt mechanisms was demonstrated by Ussing and Windhager (1964) On short circuiting of the frog skin the driving force for these shunts would be eliminated by maintaining the PD across the skin at 0 mV

c) *The pump rate might increase* as by short circuiting of the skin the active transport mechanism is facilitated when the PD across the inner barrier is decreased — Cereijido and Curran (1965) found values for the inner barrier in Rana pipiens of +52 mV in open-circuit and +18 mV in short-circuit (inside solution positive)

d) *A combination of the above mechanisms* might explain the increase in transport

Alternative (a)

If an increased influx of Na to the Na compartment were the explanation for the transepithelial transport increase on short circuiting this would result in an increase of the Na pool The present study showed no increase of the Na pool on short circuiting and this explanation therefore seems rather improbable Biber Chez and Curran (1966) made calculations of the Na pool in open circuit and short circuit conditions They used an outer solution containing 1 mM NaCl (the inside solution was ordinary Ringer s) Assuming that the Na transport pool lies in one single compartment these authors obtained a calculated increase (approximately doubled) of the Na pool on short circuiting Since these experiments were performed under completely different conditions (transepithelial osmotic difference) the results cannot be compared directly with those of the present study

Alternative (b)

Elimination of the transport by Na shunts due to short circuiting might explain the increase in transepithelial transport with retention of the Na pool in agreement with the present results

Alternative (c)

Cereijido *et al* (1964) found that the apparent Na permeability of the outer barrier decreased markedly when the Na in the outside solution was increased from 7 to 15 mM They explained their finding of a tendency to saturation of the transepithelial transport with an increasing concentration of the Na of the outside solution by this decreased permeability of the outer barrier From the results of Cereijido *et al* activation of the active transport mechanism would seem to mean a decrease of the Na pool which was not apparent in the present experiments

On the other hand later results of Rotunno *et al* (1970) have shown that the rate of flux of Na across the outer barrier to the Na compartment is high about 5 times higher than the transepithelial flux of Na Curran and Biber (Rotunno *et al* 1970) also found similar values It is evident from Fig 6 that the Na pool reached approximate tracer homogeneity after about 30 sec Since the transepithelial flux of Na has a build up half time of 2–5 min (Hoshiko and Ussing 1960), the rapid influx of Na to the Na compartment indicates that the transport compartment is saturated Activation of the active transport mechanism could then take place without a simultaneous decrease of the pool

Alternative (d)

A simultaneous increase of the influx of Na to the Na compartment and an increase of the active transport mechanism possibly in combination with elimination of shunt mechanisms might finally explain the increased transepithelial transport without a simultaneous change in the pool

These mechanisms discussed above for transepithelial transport increase without a simultaneous increase of the sodium pool are based on a model of the frog skin consisting of 2 barriers an outer passive but selective barrier and an inner barrier where the active transport mechanism is considered to be located and with an intermediate homogeneous transport compartment In recent years several findings have given reason to question the validity of this model especially with regard to the intra epithelial distribution of Na and the outer barrier

In tracer studies Cereijido and Rotunno (1967) using low Na Ringer s solution containing ^{22}Na (5—10 mM Na) on both sides of the frog skin were only able to exchange 37 % *of the total cellular Na Their conclusion was that the Na in the* epithelium lies in at least two compartments Analysis of the nuclear spin resonance of Na in frog skin (Rotunno Kowalewski and Cereijido 1967) indicated further, that only 39 % of the total Na existed in a free form as Na$^+$ ions Cereijido *et al* (1968) using ordinary Ringer s solution (115 mM Na) on both sides of the frog skin and with ^{22}Na in the outside solution were only able to exchange 12 % of the epithelial Na These authors suggested further the existence of three different compartments a non exchangeable compartment a transport compartment and an exchangeable non transporting compartment whose Na is more easily exchangeable from the inside solution but does not take part in the active transport

Results reported by Herrera and Curran (1963) and Curran Herrera and Flanigan (1963) indicate that there may be several mechanisms for flux of Na across the outer barrier They concluded that although Ca *and* ADH *both affected the outer barrier* (Ca decreased and ADH increased the flux) their actions were completely in dependent of one another

On the basis of values which they obtained for the rate of Na flux across the outer barrier (about 5 times higher than the transepithelial flux — see point (c) above) and from values for the Na pool presented in the literature Rotunno *et al* (1970) calculated the half equilibration time of the Na pool to be about 15 sec These authors on the other hand found no tendency to saturation of the uptake of Na within their observation period (46 sec) and therefore drew the conclusion that the postulated kinetic model (see above) may be erroneous

The localization of the transport compartment has been discussed and Koefoed Johnsen and Ussing (1958) have suggested that it lies intracellularly while Cereijido and Rotunno (1968) favour a localization to fixed charges in the membrane of the epithelial cells

On the basis of a model of the frog skin consisting of an outer passively but selectively permeable barrier an inner Na barrier where the active transport is considered to be located and intermediately a homogeneous Na transport compartment the result obtained in this study (that the Na pool is not changed on short circuiting) indicates that the increase of the transepithelial net flux of Na is due either to activation of the active transport mechanism (with a saturated transport compartment) or to elimination of the transport by transepithelial shunts As the Na pool was determined by continuous direct analysis the result is that the Na pool is not changed on short

circuiting regardless of which kinetic model for the sodium transport in frog skin is postulated

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A Comparison of Renal Function in Rats Anesthetized with Inactin and Sodium Amytal

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Abstract

ELMER M P C ESKILDSEN L O KRISTENSEN and P P LEYSSAC *A comparison of renal function in rats anesthetized with inactin and sodium amytal* Acta physiol scand 1972 *86* 41—58

Two distinctly different renal functional states were obtained in parallel series of non-diuretic rats anesthetized with Amytal and Inactin respectively

In the Amytal series proximal reabsorption rates ranged from 0 34 to 1 19 ml.min $^{-1}$ g KW $^{-1}$ (mean value 0 72 ml min $^{-1}$ g KW $^{-1}$) The reciprocal of the occlusion time (1/OT) and of transit time (1/TT) increased in direct proportion to the inulin clearance (Cl_{IN}) indicating that proximal luminal diameter remained unchanged over the "spontaneous range of values Proximal as well as distal reabsorption rates were independent of serum and tissue Amytal concentrations while proximal reabsorption rate was inversely correlated with log renin activity of serum

In the Inactin series proximal reabsorption rate was depressed and ranged from 0 20 to 0 74 ml min $^{-1}$ g KW $^{-1}$ (mean value 0 46 ml.min $^{-1}$ g KW $^{-1}$) 1/OT and 1/TT was constant indicating a direct relationship between luminal diameter and Cl_{IN} Proximal reabsorption rate was inversely correlated with serum Inactin concentration while no relationship between proximal reabsorption rate and log renin activity was apparent

It is concluded that the variation in proximal reabsorption rate (and filtration rate) observed in Inactin anesthetized rats is induced by the anesthetic which in some way limits the reabsorption rate The variation observed in Amytal anesthetized rats on the other hand is spontaneous i e the rate is limited by factors other than the anesthetic

In the field of renal function studies performed by micropuncture and other techniques a number of the reported discrepant results are unquestionably due to technical shortcomings (*cf* Gottschalk and Lassiter in press) There are however other discrepancies which can hardly be attributed to methodological errors The present study deals with a discrepancy of this latter type and it aims at a clarification of its cause(s)

The discrepancy in question concerns the mutual relationship between GFR (Cl_{IN}) and a number of proximal functional parameters (luminal radius end proximal tubular fluid/plasma inulin ratio transit time and occlusion time) within the so-called spontaneous range of variation in GFR

One group of investigators have consistently observed a co-variation of the proximal luminal radius and inulin clearance in non diuretic rats over the observed range of Cl_{IN} (*e g* Wahl *et al* 1967 Schnermann *et al* 1968) In accordance with this finding most groups obtain rather constant transit time values (TT) of about 10 s independent of the rates of glomerular filtration (GFR) and proximal reabsorption Also the occlusion time (OT) under this condition have been found invariant with GFR (Wahl *et al* 1967) For several years these consistent observations were taken as evidence indicating a causal relationship between the rate of proximal fluid reabsorption and the radius squared (r^2) (Gertz *et al* 1965)

A minority of investigators however have been unable to find any variation at all in proximal diameter over the spontaneous range of inulin clearance values (Leyssac 1963 Baines *et al* 1968) These groups consistently found transit time as well as occlusion time inversely correlated to inulin clearance (*e g* Bojesen and Leyssac 1969, Arrizurieta Muchnik *et al* 1969) further these later groups obtained higher end proximal tubular fluid/plasma inulin ratios (F/P_{IN} between 2 6 and 3 0) (Gottschalk and Leyssac 1967 Bojesen and Leyssac 1969) than usually reported by other investigators The internal consistency of the findings obtained by each of the investigative groups suggests the existence of two distinctly different functional states of the kidney

It has been the aim of the present study to clarify the difference(s) in experimental conditions responsible for the observed difference in functional states and this could be established also to explore the possible mechanism of this difference

It was apparent from the literature that most investigators have used the thiobarbiturate Inactin for anesthesia while Gottschalk s group and our own had used non thiobarbiturates (pentobarbital and Amytal respectively) no other difference in experimental conditions likely to be of importance could be detected from reports of the methods applied It was therefore first examined whether or not the two states of function could be reproduced in parallel series of rats anesthetized with Inactin and Amytal respectively all other conditions being equal

The results clearly show that the two characteristic functional states in question were reproduced in the two series indicating that the difference in the barbiturates administered may be the only factor causing this difference in renal tubular function Furthermore the data indicate that in Inactin anesthetized rats there is an inverse linear relationship between net proximal reabsorption rate and serum barbiturate concentration In Amytal anesthetized rats the rate of proximal reabsorption is on an average higher and unrelated to serum barbiturate concentrations Thus we are led to the conclusion that the range of spontaneous variation in proximal reabsorption rate (and GFR in Inactin anesthetized rats is artificial and limited by some function of barbiturate serum concentration (or dose) while that of Amytal anesthetized rats reflect the operation of non barbiturate limited regulatory mechanisms

Methods

Male SPF Sprague Dawley rats weighing 240—260 g were allowed free access to food and water prior to the experiment.

The rats were anesthetized with intraperitoneal injections of barbiturate.

Group I was given sodium amobarbital (sodium Amytal) 15 mg/100 g b.w. Anesthesia was supplemented or maintained when necessary with additional intraperitoneal injections of sodium Amytal. The total dose administered ranged from 15 to 20 mg/100 g b.w. over a period of about 1 1/2—2 h.

Group II was given Inactin (Promonta, Hamburg) 15 mg/100 g b.w. Since most of our rats would not sleep sufficiently for operation within 20 min on this dose, anesthesia was supplemented when necessary with additional doses of 2.5 mg until it was adequate. This series was completed by deliberately lowering as well as elevating the anesthetic dose as far as possible. The total dose administered ranged from 12 to 25 mg/100 g b.w. The experiments were carried out in two parallel series by two investigators (ME and PL) in order to see to which degree the OT—TT data would correspond when measured independently under equal conditions by two different persons.

The experimental procedure was identical for group I and II. The rats were placed on a heated operating table. Utilizing a rectal thermometer the actual body temperature controlled the heat regulation to maintain body temperature about 37° C. The rats were prepared for clearance, micropuncture and occlusion time measurements as previously described (Leyssac 1963, 1964). Polyethylene catheters were inserted into the left jugular vein for infusion of inulin and Lissamine green injections, and into the right carotid artery for blood collections and continuous recording of systemic arterial pressure.

Priming doses of 15—20 mg of inulin in 10 % solution were followed by continuous infusions of 1 % inulin in 0.9 % NaCl at a rate of 0.15 mg/min. The experiments started after an equilibration period of about 45 min. After collection of the first arterial blood sample 2 to 4 serial urine samples were collected within 15—30 min for clearance determinations. Urine samples were collected directly into calibrated polyethylene tubing (PE50) and the volume excreted determined from the length of the urine column.

Proximal luminal occlusion time (OT) was measured immediately after the last urine collection as described previously (Leyssac 1964). Transit time was measured 2—3 minutes after the OT measurement when urine flow had reached the preceding level. 75 nl 5 % Lissamine green (LG 280 X from E. Gurr, Lond.) in 0.9 % saline titrated with NaOH to pH 7.4 were rapidly injected i.v. Proximal transit time (TT_P) was measured by the modification of Steinhausen's method (1963) described by Gertz *et al.* (1965). Transit time through the loop of Henle (TT_H) was measured by subtracting TT_P from the time interval between the appearance in the capillaries and the appearance in the distal tubules.

Immediately after measurement of OT and TT and collection of the second blood sample for inulin measurements 3—4 ml of arterial blood were collected in cooled vials and kept at 0° C until centrifugation. The serum was quickly frozen (−20° C) for later measurements of renin activity and barbiturate concentration. After blood collections the left kidney was removed, drained and weighed. After weighing the kidney was frozen and kept for later measurements of barbiturate content. Inulin in plasma and urine was measured by the diphenylamine-method of Bojesen (1952) modified for microanalysis.

Systemic arterial pressure (BP) was monitored continuously throughout each experiment with a capacitance pressure transducer (Hanssen) connected to a Varian—or Servogor recorder. Intratubular pressure (ITP) was measured by the Landis method as described by Gottschalk and Mylle (1956). The pressure in the micropipette and a capacitance pressure transducer (Hanssen) was adjusted by means of a mercury leveling bulb. All connections were stiff water filled tubings. Proximal as well as distal ITP were measured in several convolutions during each urine collection period.

Measurement of renin in serum was based on the principle of determination of the decrease in angiotensinogen concentration with time (Poulsen 1969 b). The concentration of liberated angiotensin I was measured by radioimmunoassay of angiotensin I with (1-asp, 5-Ileu) angotensin I (Bio Schwarz) as standard preparation. The principle of this assay was the same as that previously described for angiotensin II (Poulsen 1969 a). The renin concentration was determined from the ratio between the initial concentration of angiotensinogen and that measured after 8 h incubation (or 24 h incubation at low renin concentrations). Renin activity expressed as the initial generation rate of angiotensin I in serum ($ng\ ml^{-1}\ h^{-1}$) was calculated from the renin concentration and the substrate concentration (= initial angiotensinogen concentration).

Amytal and Inactin were measured by a method essentially similar to the one described by Frey *et al.* (1961). The barbiturates were extracted with chloroform from acidified diluted serum samples or buffered tissue homogenates. 6 ml of the filtered chloroform phase w

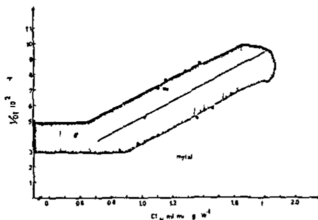


Fig 1 Relationship between inulin clearance (Cl_{IN}) and 1/OT over the range of spontaneous variations in rats anesthetized with Amytal The regression line and hatched area is taken from data previously reported (Bojesen and Leyssac 1969 mass plot)

with 0 6 ml NaOH solution (pH 12 4) in which the absorbance was measured spectrophotometrically Concentrations were measured by optical density differences Inactin from the difference between the densities at 303 and 325 μm in the alkaline solution Amytal from the difference between the density of the alkaline solution at 255 μm and that of an acidified solution at the same wavelength (after addition of 6 N HCl) Concentrations were expressed as the concentration of the sodium salt (mg %) by relating the optical density differences to standad curves The recovery was 93±3 % for serum and 96±3 % fortissue homogenate All analyses except those of the renal papilla were performed in duplicate or triplicate

Calculations

Inulin clearance was calculated from the urine/plasma inulin concentration ratio (U/P_{IN}) and the volume of urine collected per minute (V_U) divided by the kidney weight (KW) according to the conventional expression

$$Cl_{IN} = (U/P_{IN}) \quad (V_U/KW) \text{ ml min}^{-1} \text{ g KW}^{-1}$$

End proximal tubular fluid/plasma inulin ratio (F/P_{IN}) was calculated using the expression derived by Bojesen and Leyssac (1969)

$$TT_P/OT = \ln(F/P_{IN}) \qquad (^1)$$

For this calculation all values of OT were corrected for 12 % reflux from pars recta The main assumptions underlying this calculation are first that proximal fluid reabsorptive capacity is independent of the radius (load) and second that the luminal radius is identical along the convoluted part of the proximal tubule

Absolute rate of proximal reabsorption was calculated from the Cl_{IN} and F/P_{IN}

The rate of reabsorption in nephron segments distal to the proximal convolutions (lower nephron segment) was calculated as the difference between overall tubular reabsorption rate and reabsorption rate in the proximal convolutions

In the statistical analysis conventional regression analysis (least squares) was used The analysis was carried out by cand stat M Laren and cand stat Axel af Rosenborg Statistical Department Gentofte County Hospital Copenhagen Denmark

Results

a) Occlusion time—transit time

The relationship between 1/OT and inulin clearance of non diuretic rats anesthetized with Amytal (group I) is given in Fig 1 It is apparent that with few exceptions the pre ent d ta fall within the range of values obtained previously in the control functional state tate A characterized by Boje en and Leyssac 1969) The data from the few experiments falling outside the normal range were included in

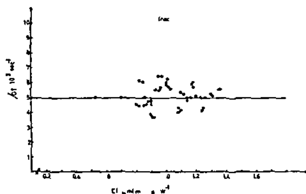


Fig 2 Relationship between inulin clearance (Cl_{IN}) and 1/OT over the range of "spontaneous" variations in rats anesthetized with Inactin The horizontal line is drawn through the mean value of 1/OT

the material because the TT/OT ratio intrabular pressures (TTP) and urine flows were within the range previously observed in Amytal anesthetized non diuretic rats From a total of 52 group I expts 12 were excluded because they did not fulfil the criteria defining the state A (*cf*[1]) a) Two expts in which TT/OT ratio was significantly below the normal range of state A and the 1/OT Cl_{IN} relationship also deviated from normal for reasons not directly apparent ITP and urine flows were within the normal range b) 2 expts in which ITP were above normal and urine flow had suddenly increased (the non steady state C Bojessen and Leyssac 1969) c) 8 expts which were technically *unsuccessful*

Fig 1 shows that 1/OT increases in linear proportion to the inulin clearance in non diuretic rats anesthetized with Amytal as previously reported Inulin clearance averaged 1 18 ml min^{-1} g KW^{-1} Proximal transit time (TT) varied in direct proportion to the OT in these experiments The TT/OT ratio and thus the F/P_{IN} ratio calculated from eq (2) remained unchanged over the range of spontaneous variations in inulin clearance (Fig 3 Amytal) The calculated F/P_{IN} ratio in state A averaged 2 62 in the present series The regression line ($y = -0\ 26 \times + 2\ 9$ S E of regress coeff 0 14) calculated from these data is not significantly different from the horizontal line (Fig 3 Amytal) drawn through the mean value ($P > 0\ 05$) The absolute rate of proximal reabsorption calculated from the inulin clearance and F/P_{IN} thus increased in direct proportion to the clearance in state A as previously observed

In non diuretic rats of group II anesthetized with Inactin 1/OT was invariant with inulin clearance over the entire range of clearance values in contrast to observations obtained in state A as apparent from Fig 2 The regression line ($y = 0\ 31 \times + 4\ 7$ S E of regress coeff 0 48 N = 74) calculated from the present data (including data from ME and PL) is not significantly different from the horizontal line drawn through the mean value of 1/OT ($p > 0\ 25$) Inulin clearance

[1] Definition of state A *Primary characteristics* Cl_{IN} 0 6 to 1 8 ml min^{-1} g KW^{-1} ITP_p 12—13 mm Hg radius 14 μ *Derived characteristics* 1/OT and 1/TT proportional to Cl_{IN} OT/TT ratio 0 9—1 0 independent of Cl_{IN}

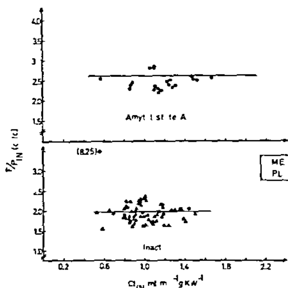


Fig 3 Calculated F/P_{IN} ratios over spontaneous variations in inulin clearance in rats anesthetized with Amytal (upper graph) and Inactin (lower graph) Horizontal lines drawn through the mean F/P_{IN} values of data above a clearance value of 0 6 ml min^{-1} g KM^{-1} ME and PL refers to data obtained by 2 operators in separate expts

averaged 0 90 ml min^{-1} g KW^{-1} a value significantly lower than that obtained in rats anesthetized with Amytal ($p < 0\,0015$) Proximal transit time was equally invariant with Cl_{IN} except for the lowest clearance values (below 0 6 ml min^{-1} g KW^{-1} corresponding to state B of Amytal anesthetized animals (Bojesen and Leyssac 1969)) At these low clearance values transit time and therefore F/P_{IN} ratio increased with decreasing clearance (4 expts cf Fig 3 Inactin) In the range of clearance values from 0 6 to about 1 6 ml min^{-1} g KW^{-1} TT/OT—and calculated F/P_{IN} ratios remained unchanged The regression line ($y = -0\,26$ $x + 2\,24$ S S of regress coeff 0 13 N = 67) calculated from the F/P_{IN} data is not significantly different from the horizontal line drawn through the mean F/P_{IN} value of 2 01 ($p > 0\,05$) (Fig 3 Inactin) and there was no difference between data obtained by ME and PL Thus within this range the absolute rate of proximal reabsorption increased in direct proportion to the clearance as was also

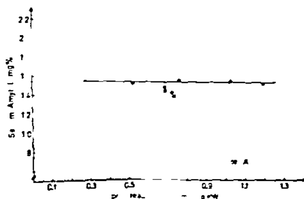


Fig 4 Serum Amytal concentration plotted against proximal reabsorption rate Horizontal line drawn through the mean Amytal concentration.

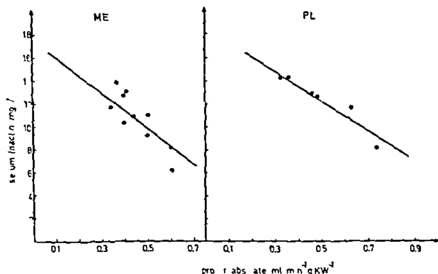


Fig 5 Relationship between serum Inactin concentration and proximal reabsorption rate Data from 2 independent series carried out by 2 operators are presented in separate graphs.

the case in rats anesthetized with Amytal (state A) However since the fraction of filtrate reabsorbed by the proximal convolution (F/P_{IN}) as well as the inulin clearance were significantly lower in group II than in group I it appears that the absolute rate of proximal reabsorption was significantly depressed in rats anesthetized with Inactin as compared to those anesthetized with Amytal

Intratubular pressures Proximal as well as distal ITP were invariant with inulin clearance in both groups of rats nor were there any difference in pressures between the 2 groups In group I (Amytal) proximal ITP was 12 8 ± 0 70 mm Hg (mean ± S D) and distal ITP was 5 8 ± 1 1 mm Hg In group II (Inactin) proximal and distal ITP were 12 96 ± 0 90 mm Hg and 6 2 ± 0 8 mm Hg respectively

Serum barbiturate

In order to analyse into further detail the cause of this difference in functional states between rats anesthetized with Amytal and Inactin serum concentrations of barbiturate at the end of the experiments were plotted against calculated proximal reabsorption rates

Fig 4 shows that in group I there was no relationship between serum Amytal concentration and the proximal reabsorption rate over the entire range of variation The regression line (y = − 1 88 × + 16 7 S E of regress coeff 1 7 N = 40) calculated from these data is not statistically different from the horizontal line drawn through the mean Amytal concentration of 15 3 mg % (p > 0 2) Proximal reabsorption rate ranged from 0 35 to 1 19 ml min⁻¹ g KW⁻¹ with a mean value of 0 72 ml min⁻¹ g KW⁻¹

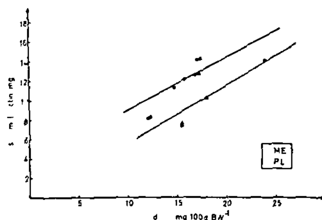


Fig 6 Relationship between Inactin concentration in serum samples collected at the end of the experiments and the dose administered intraperitoneally The regression lines were calculated from data of 2 separate series carried out by 2 operators ME and PL respectively

In contrast to this finding Fig 5 shows that in group II there was an inverse linear relationship between serum Inactin concentration and the proximal reabsorption rate This was true for 2 series carried out independently by two operators (ME and PL respectively) The regression lines given in Fig 5 ($y = -15.6x + 17.7$ S E of regress coeff 4 2 N = 21 and $y = -12.8x + 18.6$ S E of regress coeff 3 1 N = 23 respectively) are highly significant ($p < 0.005$ and $p < 0.001$ respectively) It is seen that proximal reabsorption rate ranged from 0 20 to 0 74 ml min⁻¹ g KW⁻¹ in this group with a mean value of 0 46 ml min⁻¹ g KW⁻¹ Thus the data indicate that the range of proximal reabsorption rates in rats anesthetized with Amytal was entirely independent of the anesthetic concentrations of barbiturate in serum Contrary to this proximal reabsorption rates of rats given Inactin (group II) were depressed relative to those of group I and apparently most significantly dependent upon the serum Inactin concentration It should be noted however that for any given reabsorption rate rats investigated by ME had a lower Inactin concentration in serum collected at the end of the experiment than those studied by PL This fact which will be subject to further discussion indicates that the rate limiting factor for proximal reabsorption in rats anesthetized with Inactin is not the serum barbiturate concentration itself at the moment of measurement of the reabsorption rate

This point was further analysed by plotting the serum Inactin concentration of samples collected at the end of the clearance periods versus the dose of Inactin administered in the 2 series

The data given in Fig 6 shows firstly that there was a linear relationship between the serum Inactin concentration and the dose administered with similar slope in the two series obtained by two investigators (ME and PL respectively) The regression lines ($y = 0.59x - 0.23$ S E of regress coeff 0 13 N = 21 and $y = 0.54x + 3.81$ S E of regress coeff 0 11 N = 23 respectively) were highly significant ($p < 0.001$, and $p < 0.001$ respectively) Secondly the intercepts with the ordinate are different indicating that for any given dose rats operated by ME had a lower

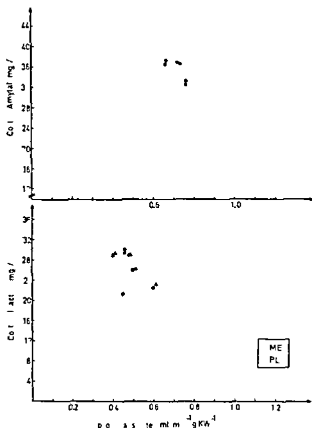


Fig 7 Cortical tissue barbiturate concentration plotted against proximal reabsorption rate Upper graph Amytal anesthesia Lower graph Inactin anesthesia

serum concentration at the end of the experiments than those operated by PL Thus viewed together the results presented in Fig 5 and 6 indicate that the rate limiting factor for proximal reabsorption in rats anesthetized with Inactin is rather some function of the dose administered than the serum concentration *eo ipso*

Since barbiturates accumulate in various tissues including the renal cortex as a function of the serum concentration (*cf* Christensen Østergaard Kristensen and Leyssac in prep) it still remained possible that proximal reabsorption rate was directly dependent upon the tissue barbiturate concentration

In Fig 7 barbiturate concentrations in the renal cortex are plotted versus proximal reabsorption rate in the two groups investigated It is apparent that even though cortex concentrations were higher than the serum concentrations there was no clear relationship between cortex barbiturate and proximal reabsorption rate A regression line ($y = 0.26X + 32.3$ S E of regress coeff ± 2 $N = 36$) calculated from the cortex Amytal concentrations and proximal reabsorption rates (Fig 7 upper graph) is not significantly different from a horizontal line through the mean value ($p > 0.5$) A regression line calculated from cortex Inactin concentrations and corresponding reabsorption rates (Fig 7 lower graph) ($y = -12.4X + 31.4$

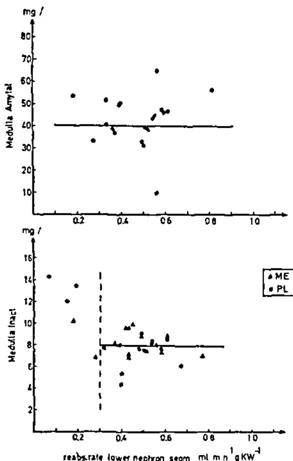


Fig 8 Medullary tissue barbiturate concentration plotted against reabsorption rate by the lower nephron segments Upper graph Amytal anesthesia Horizontal line drawn through the mean Amytal concentration Lower graph Inactin anesthesia Horizontal line drawn through the mean Inactin concentration of data above reabsorption rate of 0 3 ml min⁻¹ g KW⁻¹

S E of regress coeff 5 7 N = 43) is only on the borderline of being significantly different from zero ($0.025 < p < 0.05$) in contrast to the significant relationship between serum Inactin concentration and the reabsorption rate (*cf* above) It may therefore be concluded that the proximal reabsorption rate is not determined by the overall cortical tissue barbiturate concentration in rats anesthetized either with Amytal or with Inactin

In Fig 8 renal medullary tissue concentrations of Amytal and Inactin respectively are plotted versus reabsorption rates in the lower nephron segments (incl pars recta the loop of Henle distal tubule and collecting duct) It is seen that the lower nephron reabsorption rate was unrelated to the medullary barbiturate concentration in rats anesthetized with Amytal (Fig 8 upper graph) even though the tissue Amytal concentration was higher than the serum concentration The regression line calculated from these data ($y = 7.76x + 36.5$ S E of regress coeff 14 5 N = 36) is not significantly different from the horizontal line drawn through the mean tissue concentration of 40 0 mg % ($p > 0.6$)

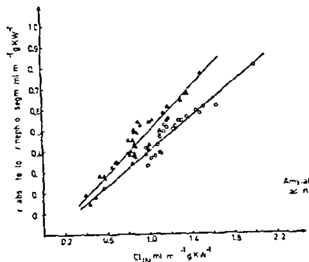


Fig 9 Relationship between reabsorption rate by lower nephron segments and inulin clearance in rats anesthetized with Amytal and Inactin respectively

In rats anesthetized with Inactin a significant correlation was found between these parameters even though the tissue Inactin concentration of the medulla was a little lower than the serum Inactin concentration ($y = -6.7x + 11.3$ S E of regress coeff 2 2 N = 46 $p < 0.005$) However it is apparent that the significance depends entirely upon the data obtained at lower nephron reabsorption rates below 0.3 ml min^{-1} g KW^{-1} When the results obtained at reabsorption rates above 0 3 ml min^{-1} g KW^{-1} were analyzed separately no correlation was detectable for these values ($y = 7.83 + 0$ x S E of regress coeff 0 03 N = 39 $p = 0.82$) Thus it would appear that reabsorption rate by the lower nephron segments is depressed by Inactin only at the highest medullary tissue (—and serum) concentrations

When reabsorption rates by the lower nephron segments in the two groups are plotted against the inulin clearance (Fig 9) it appears that at any given clearance value lower nephron reabsorption rate was higher in rats anesthetized with Inactin than in those given Amytal The regression line for group I (Amytal) ($y = 0.417 x - 0.032$ S E of regress coeff 0 02 N = 40 $p < 0.001$) is significant different ($p < 0.01$) from the line calculated for group II (Inactin ($y = 0.534x - 0.039$ S E of regress coeff 0 04 N = 46 $p < 0.001$)

d) Renin activity

Renin activity was measured in peripheral serum collected immediately after completion of the clearance OT experiments in both groups of rats Since the hypothesis has been advanced that the renin angiotensin system is directly involved in the physiological regulation of proximal tubular reabsorption rate (Leyssac 1964 1965) renin activity was plotted against proximal reabsorption rate in the two groups The results are presented in Fig 10 and 11 The data show that there was an inverse linear correlation of high significance between log renin activity and rate

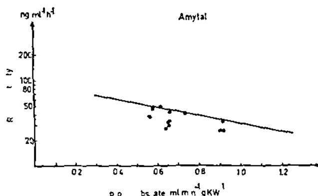


Fig 10 Relationship between serum renin activity and proximal reabsorption rate (semi log plot) of rats anesthetized with Amytal.

of reabsorption in rats anesthetized with Amytal (Fig 10) (log $y = -0.46x + 1.96$ S E of regress coeff 0 14 N = 35 $p < 0.005$) No significant relationship could be detected from data obtained in rats anesthetized with Inactin (N 35 $p > 0.1$), as apparent from Fig 11 A comparison between Fig 10 and Fig 11 indicates that the absence of a relationship in the latter is due to a more frequent occurrence of low activities at low reabsorption rates High rates of reabsorption were never associated with high renin levels neither in the Amytal nor in the Inactin group

The data were further analyzed for a possible correlation between log renin activity and reabsorption rate by the lower nephron segments No significant correlation was found in either group ($p > 0.1$)

Summarizing the results it was found that in rats of group I (Amytal) there s an inverse linear relationship between log renin activity and proximal reabsorption rate while there was no relationship between serum barbiturate concentration and the reabsorption rate The contrary was true for rats of group II (Inactin) in which there was an inverse linear relationship between serum barbiturate concentration and proximal reabsorption rate but no relationship between log renin activity and reabsorption rate

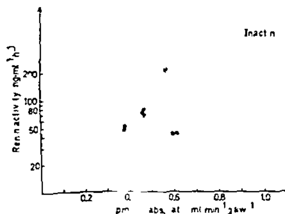


Fig 11 Semi log plot of serum renin activity versus proximal reabsorption rate of rats anesthetized with Inactin

Discussion

Inactin was introduced by Wirz (1956) as an anesthetic suitable for micropuncture studies of the renal concentrating mechanism since it was claimed to be possible to induce and maintain a water diuresis in laparotomized rats anesthetized with this thiobarbiturate this is not possible in non thiobarbiturate anesthesia (Wirz 1957) Since then Inactin has been the anesthetic used for traditional reasons in most micropuncture studies on tubular function in rats Also its administration is easy Only one single dose is required and the anesthesia will be adequate during the entire experiment for many hours without any supplement Only a few groups including Dr Gottschalk s and our own have utilized non thiobarbiturates such as sodium amytal or sodium pentobarbital It was impressive though that results obtained by these latter groups were in mutual accordance as mentioned in the introduction but disagreed in several essential respects with those published by other groups utilizing Inactin This apparent fact motivated a more detailed comparison of renal tubular function in two groups of rats anesthetized with thiobarbiturate as opposed to non thiobarbiturate but otherwise kept under equal experimental conditions

The observations by Hadfield and Ramsay (1969) suggesting that thiobarbiturates in contrast to non thiobarbiturates may inhibit the peripheral action of antidiuretic hormone in dogs and in the isolated frog skin are interesting in this context Such an effect would explain why a water diuresis may be induced more successfully in rats anesthetized with Inactin than with non thiobarbiturates

The present investigation has shown that two distinctly different states of function were obtained in the same strain of rats anesthetized with Amytal and Inactin respectively Almost all rats anesthetized with Amytal showed the same functional pattern (state A) as previously described (*cf* Bojesen and Leyssac 1969) in which 1/OT (as well as 1/TT) increases in direct proportion to the inulin clearance The F/P_{IN} ratio as estimated from the TT/OT ratio remained unchanged in this series at a value of about 2 6 over the entire range of spontaneous clearance values thus the absolute rate of proximal reabsorption increased in direct proportion to the GFR and may be estimated directly from the reciprocal of the occlusion time in this state of function This implies that the proximal luminal diameter is the same over the entire spontaneous range of clearances Luminal diameters measured directly in enlarged photomicrographs of freeze dryed cryo-sections of the kidneys from the same animals snap frozen at the end of the experiment confirmed this inference (Elmer Østergaard Kristensen and Leyssac manus in prep) in close agreement with previous findings in rats anesthetized with Amytal (Leyssac 1963 Baines *et al* 1968) All data from the amytal state A thus are quantitatively consistent and in close agreement with previous data obtained in rats under the same conditions and anesthesia It should be mentioned however that the state A in Amytal anesthesia is rather labile and may easily be disturbed by interference with the experimental conditions In the present series of 52 expts state A

not maintained in 12 expts in 8 of these for obvious technical reasons (ureteral obstruction inadequate or too deep anesthesia, failure in the temperature control)

Almost all the rats anesthetized with Inactin showed an identical functional pattern apparently less sensitive to occasional minor interferences with the experimental conditions In the Inactin induced state of function absolute rate of proximal reabsorption also increased in direct proportion to the inulin clearance while the occlusion time (as well as the transit time) was invariant with the clearance in agreement with the data reported by Wahl *et al* (1967) Thus proximal luminal diameter must have increased with GFR Direct measurements of the diameters in freeze-dryed cryo-sections from the same experimental kidneys snap frozen at the end of the experiment confirmed this inference The radius increased from 12 μ at the low rates of filtration (0 4 ml min^{-1} g KW^{-1}) to reach the value of 14 μ observed in Amytal anesthetized rats at filtration rates of 1 5 ml min^{-1} g KW^{-1} (Elmer Østergaard Kristensen and Leyssac manus in prep) The slope of the regression line was not significantly different from that reported by Wahl *et al* (1967) in similarly anesthetized rats Further an F/P_{IN} ratio of about 2 0 in the present series calculated from the TT/OT ratio agrees quantitatively with values measured directly in collected end proximal fluid samples from Inactin anesthetized rats of other investigative groups (*e g* Brenner *et al* 1968 1971 Landwehr *et al* 1968 Lowitz *et al* 1968 Spitzer and Windhager 1970) Thus using our methods we have obtained results which agree closely with those obtained by other groups using the same anesthetic and the same as well as other techniques For this calculation OT values were corrected for 12 % reflux as was found in state A (Bojesen and Leyssac 1969) Reflux was not measured in rats of group II (Inactin) and Wahl et al (1967) did not observe reflux in their rats, while Brandis *et al* (1967) did in rats similarly anesthetized with Inactin Thus proximal reabsorption rate is possibly slightly overestimated in the present calculations from data collected in Inactin anesthetized animals Without any correction for reflux the mean F/P_{IN} ratio would be 1 86 for the Inactin group

Both barbiturates may depress cellular functions including transepithelial transport of solutes and water (Christensen Østergaard Kristensen and Leyssac manus in prep) Thus it remained possible that the spontaneous range of proximal reabsorption rates (and thereby of GFR s) in both functional states were artificial and determined by the range of barbiturate concentrations necessary to establish the adequate anesthetic level However the present study clearly shows that proximal as well as distal reabsorption rates were independent of the barbiturate concentration in both serum and renal tissue of rats under Amytal anesthesia In contrast proximal reabsorption rate (and GFR) was significantly depressed in Inactin anesthesia to a mean value of 0 46 ml min^{-1} g KW^{-1} as opposed to 0 72 ml min^{-1} g KW^{-1} in Amytal anesthesia furthermore the rate of proximal reabsorption correlated most significantly with serum Inactin concentration being the more depressed the higher the serum concentration

The isosmotic fluid transport process by the gall bladder *in vitro* is qualitatively

as well as quantitatively similar to that of renal proximal tubules The present finding is therefore strongly substantiated by results from a parallel in vitro-study indicating that Inactin is a far more potent inhibitor of isosmotic net fluid transport by the gall bladder than Amytal and that net transport in vitro is depressed irreversibly by medium Inactin concentration levels equal to those measured in plasma of anesthetized rats In contrast medium Amytal concentration levels equal to those in plasma of anesthetized rats were without any inhibitory effect on in vitro transport by the gall bladder (Christensen Østergaard Kristensen and Leyssac manus in prep)

It seems therefore a valid conclusion that the spontaneous range of proximal reabsorption rates and inulin clearances in rats anesthetized with sodium Amytal is not limited directly by the anesthetic itself the range rather seems to reflect the operation of endogenous regulatory mechanisms In Inactin anesthesia on the other hand proximal reabsorption rate is directly depressed and limited by the barbiturate Therefore under this anesthesia the spontaneous range of variation of proximal reabsorption rates and clearances must be considered an artifactual direct drug effect This would also explain why most investigators using Inactin have reported not only lower values but also a narrower range of reabsorption rates and clearance values than those obtained by the present group This would be the simple consequence of using a standard dose of Inactin per g of b w The relatively large range of reabsorption rates obtained in the present Inactin series was deliberately provoked by lowering as well as elevating the dose within the limits permitting an adequate anesthesia for operation and normal blood pressure and transit times

An interesting detail of the Inactin effect was obtained by the observation that the effect cannot be ascribed to a direct function of the plasma concentration at the moment of measurement of the transport rate For any given dose of Inactin (per g b w) rats anesthetized by one of the present investigators (ME) had a lower serum barbiturate concentration at the end of the experiment than those anesthetized and operated by the other (PL) The duration of the experiments was almost the same for the two investigators The finding may probably be explained by a difference in the technique of intraperitoneal administration of the anesthetic solution (cranial versus caudal injection into the abdomen) resulting in a faster absorption and thus higher initial plasma concentration in the case of ME s animals due to his more cranial injections Since tissue uptake (accumulation) is a function of the plasma concentration tissue uptake and thereby elimination from plasma would be faster initially in rats anesthetized by ME this would finally result in a lower serum concentration at the end of the experiment (about 2 h) Further for any given rate of reabsorption ME found a lower serum Inactin concentration than the other investigator Thus the lower serum concentration was without influence on the depression of tubular function obtained by a given dose Therefore taking into account the irreversibility of the Inactin effect on fluid transport the present data indicate that the rate limiting factor for proximal reabsorption under *

anesthesia is a function of a time integral of the plasma concentration rather than a direct function of the Inactin concentration itself

Three observations viewed together suggest that the distal epithelium is less sensitive to Inactin than the proximal epithelium and proceeds at a transport rate corresponding to a higher (non inhibited) level of GFR and rate of proximal reabsorption Firstly lower nephron reabsorption rates in most experiments were in the same range in the Amytal and Inactin groups in contrast to the difference between the observations on proximal reabsorption rates and secondly for a given inulin clearance the reabsorption rate was higher in Inactin than in Amytal anesthetized rats thirdly above rates of lower nephron reabsorption of 0 3 ml min^{-1} g KW^{-1} there was no relationship to medullary tissue Inactin concentration indicating that reabsorption rate in distal segments was uninfluenced by Inactin However at the lowest range of lower nephron reabsorption rates (below 0 3 ml min^{-1} g KW^{-1}) the data suggest an inverse relationship between medullary Inactin concentration and rate of reabsorption This would suggest that at the highest serum and medullary tissue concentrations also distal segments may be depressed. Consequently GFR would be more significantly reduced in these experiments At these concentrations one would anticipate that a stimulating effect on distal sodium transport by vasopressin would be abolished or reduced this would agree with the previously mentioned results obtained by Hadfield and Ramsay (1969)

The hypothesis has been advanced that the renin angiotensin system is directly involved in the physiological regulation of proximal reabsorption rate (Leyssac 1964 1965) Data from OT-experiments gave evidence in favour of this concept which also motivated the present measurements of serum renin activity The question whether or not angiotensin inhibits proximal reabsorption rate has been considered an open question since Burg and Orloff (1968) were unable to find any significant effect of angiotensin on net absorption rate by isolated proximal tubular segments in vitro However the absorption rates obtained in vitro were lower than the lowest spontaneous rates observed in Amytal anesthetized rats in vivo The negative findings in proximal segments in vitro by Burg and Orloff therefore cannot be considered critical Results from in vivo experiments on rats using the OT as well as the split oil drop method have also failed to reproduce the original observations (*e g* Thurau *et al* 1967 Lowitz *et al* 1969) These results were obtained in rats anesthetized with Inactin and the split oil drop method in its original version is too inaccurate because of recognized inborn errors (*cf e g* Nakajima *et al* 1970 Györy 1971) These latter observations therefore cannot either be any argument against the hypothesis nor the results upon which it was based In support of the observation that angiotensin may inhibit fluid transport rate is the report of such an effect on transport by the gall bladder (Frederiksen and Leyssac 1969) and by the stripped colonic epithelium Davies Munday and Parsons 1970)

In the present experiments an inverse linear correlation was found between log renin activity and spontaneous proximal reabsorption rate in rats anesthetized with Amytal Even though this correlation nicely fits the above mentioned hypothesis it

cannot of course be taken as any proof Such a relationship might be claimed to be a co variation with depression of renal function in general However if this were the actual case one would predict a similar correlation when renal function was depressed by Inactin since the presence in this functional state of so-called tubulo-glomerular balance with normal intratubular pressures indicates a proportional depression of glomerular capillary pressure The disappearance of the correlation in Inactin anesthetized rats with artificially limited reabsorption rate then would seem to lend supporting evidence in favour of the hypothesis that the renin angiotensin system is involved in regulation of proximal reabsorption rate

Results from a large fraction of experimental renal research over the last decade have been obtained in rats anesthetized with Inactin The recognition obtained from the present study urges a revision of the data obtained under this condition since their relationship to normal (physiological) renal function is questionable

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Facilitation from Ipsilateral Primary Afferents of Interneuronal Transmission in the Ia Inhibitory Pathway to Motoneurones

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Abstract

FEDINA L and H HULTBORN *Facilitation from ipsilateral primary afferents of interneuronal transmission in the Ia inhibitory pathway to motoneurones* Acta physiol scand 1972 *86* 59—81

The effects on transmission in the Ia inhibitory pathway to motoneurones by volleys in ipsilateral primary afferents were investigated with intracellular recording from motoneurones In spinal cats under chloralose anesthesia Ia IPSPs in flexor as well as extensor motoneurones were regularly facilitated by volleys in cutaneous high threshold muscle and joint afferents In decerebrate cats with a low pontine lesion IPSPs were not facilitated from high threshold muscle and joint afferents although volleys in low threshold cutaneous afferents evoked a facilitation also in this preparation It is postulated that the Ia inhibitory interneurones receive excitatory actions from the ipsilateral flexor reflex afferents (transmission depressed in the decerebrate state) and through a separate pathway from low threshold cutaneous afferents The recurrent effects from motor axon collaterals were investigated on inhibitory transmission to motoneurones from different afferents A strong positive correlation was revealed between recurrent depression of IPSPs evoked from different afferents and facilitation of Ia IPSPs by the same afferent volleys This suggests that the recurrent depression of IPSPs from different primary afferents depends on an excitatory convergence from them onto the Ia inhibitory interneurones which mediate at least part of the IPSP evoked in the motoneurone from these afferents

Antidromic impulses in motor axons depress transmission in the Ia inhibitory pathway to motoneurones by a postsynaptic inhibition of the Ia inhibitory interneurones evoked via α motor axon collaterals and Renshaw cells (Hultborn Jankowska and Lindstrom 1971 a b)—henceforth referred to as recurrent depression of Ia IPSPs Although the most striking result in the investigation by Hultborn *et al* (1971 a) was the efficient recurrent depression of Ia IPSPs in all species of motoneurones they reported that also some IPSPs evoked from the ipsilateral flexor reflex afferents (FRA) were slightly depressed by volleys in ventral roots IPSPs from Ib afferent were on the other hand never affected The recurrent depression of the FRA IPSP

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was tentatively explained by an excitatory convergence from the FRA onto the Ia inhibitory interneurones which were assumed to mediate at least part of the FRA IPSP recorded in the motoneurones. If this interpretation is correct then it would be expected that volleys in afferents which evoke an IPSP susceptive to recurrent depression also should facilitate transmission in the Ia inhibitory pathway. With this background we decided to compare the recurrent depression of IPSPs evoked from different primary afferents with the facilitation of Ia IPSPs evoked by the same afferent volleys. It will be shown that Ia inhibitory transmission to motoneurones is facilitated from the ipsilateral FRA pathway and also through a separate neuronal pathway from low threshold cutaneous afferents. Measurements from a large number of motoneurones have revealed a strong positive correlation between these facilitatory effects and the susceptibility to recurrent depression of the IPSPs evoked in motoneurones from the same afferents. Similar results were obtained in an investigation on recurrent depression of IPSPs from the contralateral FRA and *effects from these afferents on the transmission in the Ia inhibitory pathways* (Fedina and Hultborn 1972). Our results thus support the suggestion by Hultborn *et al.* (1971 a) that the recurrent inhibition of interneurones in afferent pathways to motoneurones is restricted to those mediating the reciprocal Ia inhibition to motoneurones.

The present conclusions have been of importance for the interpretation of the effects from motor axon collaterals on inhibitory transmission to motoneurones from supraspinal systems (Hultborn and Udo 1972 b).

Methods

The experiments were performed on 20 cats operated under ether anesthesia. Subsequently they were intercollicularly decerebrated or in a few cases anaemically decorticated (Andén *et al.* 1966 a) and then kept either unanesthetized or under chloralose anesthesia (40—60 mg/kg). All cats were paralyzed with gallamine triethiodide (Flaxedil, May and Baker Ltd.) and artificially respired. The maintenance of the preparation was otherwise as described previously (Hultborn *et al.* 1971 a).

Laminectomies were performed at the lower thoracic and at the lower lumbar level (L4—L7). Dorsal roots were left intact while L5—S1 ventral roots were cut and their proximal ends mounted on electrodes for stimulation or recording. The L6 and L7 ventral roots were usually divided into two halves, rostral and caudal, and stimulated separately. Muscle and cutaneous nerves and a nerve branch from the knee joint in the ipsilateral (left) hindlimb were dissected and mounted on electrodes for stimulation. These nerves are listed under Abbreviations.

The 4th ventricle was exposed by a complete cerebellectomy or in a few cases by removing only its vermal part. A brain stem lesion (at low pontine level) was then made with a 3 mm wide spatula perpendicularly from the floor of the ventricle through the entire medial brain stem as described by Holmqvist and Lundberg (1961). To avoid the very high excitability of motoneuron [illegible] a pontine lesion the ventral quadrants were mostly sectioned at the lower thoracic level (*cf.* Holmqvist and Lundberg 1961). Besides the lower excitability no differences [illegible] results could be detected after the ventral spinal lesion. The dorsal column was alw[illegible]ected at lower thoracic level. In some experiments the spinal cord was completely transected.

These procedur[illegible]lted in 4 different preparations: (i) unanesthetized decerebrate decerebellate cats w[illegible] low pontine lesion—henceforth called unanesthetized low pontine preparation, (ii) [illegible] but with chloralose anesthesia, (iii) unanesthetized spinal animals, (iv) spinal [illegible] with chloralose anesthesia. Some of the spinal animals (belonging to group iii or iv) we[illegible] [illegible]rebrated but anaemically decorticated (*cf.* above).

The techniques of intracellular recording from motoneurones (including averaging facilities) and of stimulation of nerves have been described in a previous paper (Hultborn and Udo 1972 a) The recorded intracellular potentials were shown to be due to postsynaptic potentials by their potential dependence and/or by a comparison with the extracellular field potentials Differentiation between afferent fibre groups was done as described by Bradley and Eccles (1953) and Eccles Eccles and Lundberg (1957) The conditioning shock stimulation of the ventral roots was supramaximal for α fibres and preceded the test response with 6—20 ms The stimulus repetition rate was about 1/s

Abbreviations The following abbreviations are used anterior biceps and semimembranosus ABSm deep peroneus (without cutaneous and extensor digitorum brevis branches) DP gastrocnemius and soleus G S posterior biceps and semitendinosus PBSt extensor digitorum longus EDL flexor digitorum and hallucis longus FDL posterior knee joint nerve J plantaris Pl quadriceps Q saphenus Saph soleus Sol superficial peroneal SP suralis Sur tibialis (after giving branches to popliteus tibialis posterior and flexor digitorum and hallucis longus muscles) Tib flexor reflex afferents FRA ventral root VR postsynaptic potential PSP excitatory postsynaptic potential EPSP inhibitory postsynaptic potential IPSP recurrent inhibitory postsynaptic potential RIPSP dorsal root potential DRP primary afferent depolarization PAD afterhyperpolarization AHP

Results

The effects by volleys in high threshold muscle high threshold joint and cutaneous afferents on transmission in the Ia inhibitory pathways to different species of motoneurones are summarized in Table I and III The recurrent control from motor axon collaterals on transmission in inhibitory pathways to motoneurones from these afferents was also tested and in individual motoneurones correlated with the facilitation of Ia inhibitory transmission evoked from the same afferents (Table II and IV) It was important that these various afferent volleys did not cause any firing of motoneurones in order to exclude that a depression of transmission in the Ia inhibitory pathway or that IPSPs evoked in motoneurones by these volleys were secondary to impulses in motor axon collaterals and Renshaw cells We therefore regularly controlled whether the afferent volleys used elicited a discharge in the ventral roots

High threshold muscle afferents high threshold joint afferents and cutaneous afferents have many central actions in common and are often grouped together as the flexor reflex afferents (FRA R M Eccles and Lundberg 1959 a) We will describe separately the effects from these afferent systems and show that some results from cutaneous afferents differ from those evoked from high threshold muscle and high threshold joint afferents

High threshold muscle afferents

Spinal chloralose anesthetized preparation In acute spinal cats volleys in the ipsilateral FRA give dominating excitation to flexor and inhibition to extensor motoneurones (R. M Eccles and Lundberg 1959 a) We were therefore surprised to find that volleys in high threshold muscle afferents facilitated transmission in the Ia inhibitory pathway both to flexor and extensor motoneurones and that the effect on the pathway to flexor motoneurones seemed to be more pronounced However it is recalled that the ipsilateral FRA seem to have access to alternative excitatory and inhibitory pathways to both flexor and extensor motoneurones (R. M Eccles

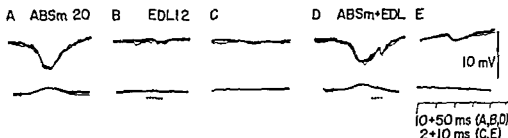


Fig. 1. *Facilitation from high threshold muscle afferents of transmission in the Ia inhibitory pathway to an ankle extensor G.S. motoneurone.* Upper traces are intracellular potentials and consist of several superimposed sweeps. Lower traces are recorded from L7 dorsal root entry zone. In this and the following figures positivity in intracellular and negativity in surface recordings are signalled upwards. Stimulation strength of peripheral nerves is expressed in multiples of threshold for the lowest threshold fibres. The unconditioned and conditioned test Ia IPSPs were taken simultaneously at a slow and fast sweep speed. The dashed lines below the slow records in B and D show the parts which are expanded in C and E respectively. Time calibration as indicated below E. Voltage calibration refers to intracellular potentials. Chloralose anesthetized spinal cat.

and Lundberg 1959 a, Holmqvist and Lundberg 1961) and that inhibition sometimes is the dominating effect also in flexor motoneurones (R. M. Eccles and Lundberg 1959 a).

Fig. 1 illustrates an ankle extensor G.S. motoneurone. Records in B (and with faster sweep speed in C) show the minute test Ia IPSP evoked by stimulation of the nerve from the EDL muscle. A conditioning volley in the ABSm nerve (20× threshold) gave a *facilitation of the test Ia IPSP (D—E) thereby proving the excitatory action from these high threshold muscle afferents on Ia inhibitory interneurones.* This form one of the better examples of an excitatory convergence from high threshold muscle afferents on Ia inhibitory interneurones impinging on extensor motoneurones—the action was usually weaker or especially in Q motoneurones impossible to demonstrate (column 4 in Table I).

The knee flexor PBSt motoneurone in Fig. 2 was recorded in an experiment by Hultborn and Udo (preparation described in legend) in which volleys in the FRA evoked mainly inhibition in flexor motoneurones instead of excitation (*cf.* above). The small test Ia IPSP (A—B) was greatly enhanced by the preceding volley in muscle group II afferents (C—D) (a volley in group I afferents had no effect). A conditioning ventral root stimulation (E—F) virtually abolished the facilitated Ia IPSP thus showing the convergence of excitation from high threshold muscle afferents and inhibition from motor axon collaterals on the same Ia inhibitory interneurones (for a more elaborate description of this testing technique see Hultborn and Udo 1972 a). The records in G—H show that the high threshold IPSP (Sart 5.0× thresh. G) was effectively depressed by a preceding ventral root volley (H). In this motoneurone the FRA IPSP appeared as susceptive to recurrent depression as the Ia IPSP (records in I—J). Provided that the recurrently depressed parts of IPSPs in fact are mediated by the Ia inhibitory interneurones this finding would

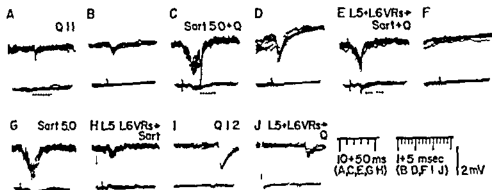


Fig 2 *Facilitation of a Ia IPSP in a knee flexor PBSt motoneurone by volleys in high threshold muscle afferents and concomitant recurrent depression of the IPSP evoked from the same high threshold muscle afferents* Upper traces are intracellular potentials Lower traces are from the L7 dorsal root entry zone The dashed lines below the slow records in A C and E show the parts which are expanded in B D and F respectively Time calibrations as indicated Voltage calibration refers to intracellular potentials Chloralose anesthetized cat The spinal cord was transected with exception of the ipsilateral ventral quadrant Intact brain

indicate that the FRA IPSP in flexor motoneurones to a large extent were conveyed by these interneurones

Current passage through the recording microelectrode used to analyse a possible mixture of excitatory and inhibitory PSPs revealed that in flexor motoneurones volleys in the ip ilateral FRA usually evoked an IPSP in addition to the dominat ing EPSP It therefore proved possible to study the facilitation of Ia IPSPs by volleys in high threshold muscle afferents as well as the recurrent depression of IPSPs from the same afferents in flexor motoneurones even in normal spinal cats with prevailing FRA excitation The motoneuronal membrane just had to be de polarized in order to amplify the IPSPs at the expense of the EPSPs

In the PBSt motoneurone illustrated in Fig 3 volleys in high threshold muscle afferents evoked dominating excitation (A) The motoneuronal membrane was then depolarized (70 nA) The FRA EPSP (A) was virtually nullified and an IPSP revealed (B) The recurrent depression of the Ia IPSP is shown in C—D The two upper traces in E are the responses to separate stimulation of high threshold muscle afferents (Pl 9 0×thresh) and of Ia afferents in the nerve from the antagonistic Q muscle (1 02×thresh) respectively When they were stimulated together the Ia IPSP was effectively facilitated A preceding conditioning ventral root stimulation (lowermost trace in E) almost abolished the facilitated Ia IPSP This proved the convergence of excitation from high threshold muscle afferents (mediated via spinal pathways) and re urrent inhibition on the same Ia inhibitory interneurones Records in F—G show that the IPSP evoked by the volley which facilitated the test Ia IPSP was depressed by preceding antidromic volleys (cf the superimpo ed traces of un conditioned and conditioned IPSPs in G)

TABLE I. Facilitation of Ia IPSPs by volleys in ipsilateral high threshold muscle afferents. The species of motoneurone investigated are indicated to the left and the figures in parentheses indicate the number of cells tested. The types of preparation are given in the upper horizontal row, column 1–4. Note that the preparations labelled decerebrate refer to decerebrate decerebellate cats with a low pontine lesion as described in Methods. The figures presented for each combination show the numbers of motoneurones in which a spatial facilitation was established of the total number of motoneurones tested in that combination (convergence/total). The sum of the motoneurones recorded in different preparations is sometimes greater than the number given in the parentheses to the left in the table. This depends on that in a few cases individual motoneurones were recorded in more than one type of preparation. Two PBSt motoneurones were thus recorded before and after spinalization in chloralose anesthetized cats—no facilitation was seen in the low pontine state but appeared in both motoneurones after spinalization. Three motoneurones (1 GS, 1 DP and 1 PBSt) were recorded first in spinal unanesthetized preparations and then after chloralose administration. In all three motoneurones chloralose administration revealed a facilitation of Ia IPSPs which was absent in the unanesthetized state.

			1 decerebrate unanesthetized	2 decerebrate chloralose	3 spinal unanesthetized	4 spinal chloralose
PBSt	(47)	convergence/total	0/14	0/7	1/6	2?/24
DP	(12)		0/3	–	0/5	5/6
ABSm	(9)		–	–	0/4	5/5
Q	(37)		0/9	0/5	0/15	4/9
GS	(20)		0/1	0/2	4/11	7/7

The facilitatory action on transmission in the Ia inhibitory pathway by volleys in *high threshold muscle afferents could sometimes be produced with a stimulus* strength of about 3—4 × threshold but more often required a strength of about 10 × threshold or even more. The facilitatory effect on the Ia IPSPs was then usually seen to grow with increasing stimulus strength up to 20—30 (—50) × threshold. These figures would thus indicate that activity in group II as well as group III muscle afferents may exert the facilitatory action on transmission in the Ia inhibitory pathway as would be expected if this facilitatory action was mediated via the FRA pathway (R. M. Eccles and Lundberg 1959 a). Facilitation of transmission in the Ia inhibitory pathways to flexor and extensor motoneurones could be evoked equally well by volleys in high threshold muscle afferents from flexors and extensors.

It should be noted that the observations reported above were made on spinal *cats anesthetized with chloralose. In the next section it will be shown that facilitation* of Ia inhibitory transmission from high threshold muscle afferents is rare in unanesthetized spinal cats.

Decerebrate cats with a low pontine lesion. A suppression of FRA actions is seen in the decerebrate state (R. M. Eccles and Lundberg 1959 b), but after a medial low pontine lesion there is a release of inhibitory paths from FRA to both extensor and flexor motoneurones while transmission in the excitatory paths is still suppressed (Holmqvist and Lundberg 1961). This latter preparation, below referred to as the low pontine preparation, seemed to offer excellent possibilities for an analysis of the inhibitory FRA path to flexor motoneurones. The PBSt motoneurone in Fig. 4 A—C was recorded in an unanesthetized low pontine preparation. To our surprise the

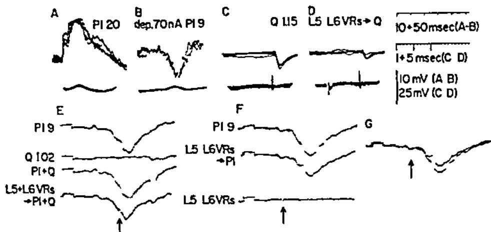


Fig 3 *Comparison of susceptibility to recurrent depression of a high threshold muscle IPSP and ability by a volley in the same afferents to facilitate a Ia IPSP in a knee flexor PBSt motoneurone* Upper traces in A—D are intracellular potentials and lower traces are recorded from L6 dorsal root entry zone All traces in E—G are averaged intracellular responses (20 responses cf Methods in Hultborn and Udo 1972 a) The time of stimulation of L5 and L6 ventral roots is marked by arrows in E—G Time and voltage calibrations for A—D are as indicated to the right of D The calibration pulses in the beginning of all traces in E—G have an amplitude of 1 mV and a duration of 4 ms The motoneuronal membrane was depolarized by a current of 70 nA during records B—G There was no evidence that this depolarizing current caused reversal of EPSPs The threshold for a ventral root discharge was at PI 10×thresh and it is thus concluded that the IPSP evoked by stimulation of PI 9×thresh was secondary to firing of motoneurones Chloralose anesthetized spinal cat

IPSP evoked by a volley in G S high threshold afferents remained unaltered (B—C) when conditioned by a volley in the same ventral roots which effectively depressed the Ia IPSP in the same motoneurone (lower traces in A) The record in A show that the same G S volley did neither facilitate the Ia IPSP from the Q nerve—on the contrary the Ia IPSP is depressed

A reduction of the amplitude of the test Ia IPSP by volleys in high threshold muscle afferents was very common in the low pontine preparation It was often possible to exclude that potential or conductance changes in the motoneuronal membrane were responsible for the decreased amplitude The depression of the test IPSPs was thus observed at conditioning testing intervals exceeding the duration of the PSPs evoked by the conditioning volley At these intervals there was no change in the input resistance measured by injecting small current pulses through the microelectrode (Eide 1968) These results indicate that volleys in high threshold muscle afferents depress transmission in the Ia inhibitory pathway to motoneurones in decerebrate cats with a low pontine lesion Also extensor motoneurones were investigated in this preparation As for flexor motoneurones it was found that volleys in high threshold muscle afferents did not facilitate Ia IPSPs (but often caused a depression of them) and that IPSPs evoked from the same afferents were unaffected by antidromic volleys (column 1 in Table I) When these unanesthetized low

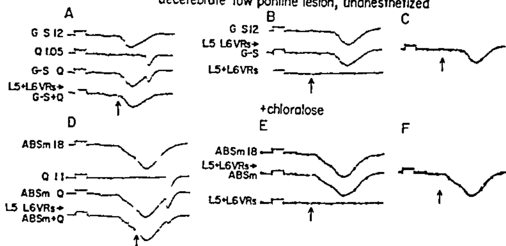


Fig 4 *Comparison of susceptibility to recurrent depression of high threshold muscle IPSPs and ability by volleys in the same afferents to facilitate Ia IPSPs* Two knee flexor PBSt motoneurones were recorded in a low pontine preparation—the first in an unanesthetized state (A—C) and the second under chloralose anesthesia (D—F) All traces are averaged intracellular responses In C and F the conditioned and unconditioned IPSPs from high threshold muscle afferents (also shown in B and E respectively) are superimposed The time of stimulation of ventral roots is marked by arrows Further description in the text The calibration pulses in the beginning of all traces have an amplitude of 1 mV and a duration of 4 ms The motoneurone illustrated in A—C was depolarized by a current of 30 nA and that in D—F by a current of 25 nA

pontine cats were spinalized it turned out that volleys in high threshold muscle afferents were still unable to facilitate Ia inhibitory transmission Column 3 in Table I shows that facilitation of IPSPs in flexor as well as in extensor motoneurones was infrequent in the unanesthetized spinal state Chloralose administration was needed to reveal a regular facilitation in spinal cats (*cf* columns 3 and 4 in Table I) and the question then arose whether the failure to facilitate Ia IPSPs in the unanesthetized low pontine state was due to absence of chloralose anesthesia Records D—F in Fig 4 from a PBSt motoneurone recorded in a low pontine chloralose anesthetized preparation demonstrate however that there was neither facilitation of the test Ia IPSP by an ABSm volley (D) nor any recurrent depression of the ABSm IPSP (E—F) Together with the results in column 2 Table I the above notion can be refuted—the lack of facilitation of Ia IPSPs by volleys in high threshold muscle afferents in the unanesthetized low pontine state is not due only to the absence of chloralose anesthesia

Recurrent depression correlated with facilitation of Ia IPSPs The susceptibility to recurrent depression of IPSP evoked by volleys in high threshold muscle afferents was compared with the ability by volleys in the same afferents to facilitate Ia IPSPs in 68 motoneurones (35 PBSt 26 Q 7 DP The results are summarized in Table II in which for individual motoneurones the recurrent depression of an IPSP from high threshold muscle afferent (ordinate) is plotted against the ability by volleys

TABLE II Comparison between the susceptibility to recurrent depression of IPSPs from high threshold muscle afferents and the ability by volleys in the same afferents to facilitate transmission in the Ia inhibitory pathway For each individual motoneurone (35 PBSt and 26 Q) the susceptibility to recurrent depression of IPSPs from high threshold muscle afferents has been plotted against the ability by volleys in the same afferents to facilitate a test Ia IPSP Different symbols indicate the type of preparation Note that the preparations labelled decerebrate refer to decerebrate decerebellate cats with a low pontine lesion as described in Methods Notice that the middle groups (both at the abscissa and ordinate) means that it was not possible to decide if the effect was present or absent — no decision

PBSt moton

	no fac. of Ia IPSP		fac. of Ia IPSP
rec. depr			●●●● ●●●● ●●●●○
	×	▽ ●	
no rec. depr	×××× ×××× ×××× ○○○○ ▽▽▽		

Q moton.

	no fac. of Ia IPSP		fac. of Ia IPSP
rec. depr	●		●●●
	× ▽●▽ ×	● ▽	
no rec. depr	×××× ××× ○○○○ ▽●●●		

× decerebrate nonesthetized
▽ decerebrate, chloralose
○ spinal unanesthetized
● spinal chloralose

in the same afferents to facilitate Ia IPSPs (abscissa) Each sign represents one motoneurone and the table gives only the presence or absence of a recurrent depression and of a facilitation of a test Ia IPSP In several cases it was however not possible to decide to which of these groups a certain result should belong and they should consequently be omitted from further comparisons They are however presented as a middle group in order to illustrate the frequency at which a decision in any direction was impossible

The PBSt material in the left part reveals that there is a strong positive correlation between susceptibility to recurrent depression and ability to facilitate Ia IPSPs There was neither facilitation of Ia IPSPs nor any depression of the high threshold muscle IPSPs by ventral root volleys in low pontine cats (unanesthetized or with chloralose anesthesia) In spinal chloralose anesthetized animals volleys in these afferents on the contrary did facilitate Ia IPSPs and their IPSPs were consequently depressed by antidromic volleys A strict positive correlation between recurrent depression and facilitation of transmission in the Ia inhibitory pathway was seen also in the 7 DP motoneurones tested (not given in Table II)

The results for the Q material presented in the right half in Table II may seem less clear since conclusions were impossible to reach in seven motoneurones Among the remaining motoneurones which could be properly judged there is however an evident positive correlation The three Q motoneurones in which a facilitation of the test Ia IPSP was seen in combination with a recurrent depression of the IPSP from the high threshold muscle afferents were all sampled in the spinal chloralose

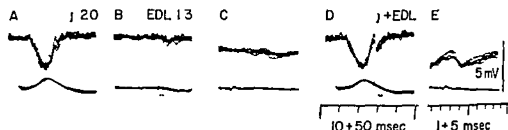


Fig. 5. *Facilitation from high threshold joint afferents of transmission in the Ia inhibitory pathway to a G.S. motoneurone.* Upper traces are intracellular responses. Lower traces are recorded from L7 dorsal root entry zone. The dashed lines below the slow records in B and D show the parts which are expanded in C and E respectively. Time calibration for the slow records in A, B and D is below D and for the fast records in C and E below E. Voltage calibration refers to intracellular potentials. Chloralose anesthetized spinal cat.

anesthetized state. However, in several Q motoneurones in the same type of preparation we obeserved no facilitation of Ia IPSPs and then usually (one exception) neither any recurrent depression of the corresponding IPSP. In low pontine cats volleys in high threshold muscle afferents did not facilitate the Ia IPSPs and mostly there was no recurrent depression of the IPSPs evoked by the same volleys. A clear recurrent depression of the high threshold muscle afferent IPSP without any corresponding facilitation of test Ia IPSPs was found in one Q motoneurone (left upper square in Table II). This IPSP was not secondary to firing of motoneurones as judged by the lack of any discharge in ventral roots. Possible explanations for such deviations from the expected positive correlation between recurrent depression and ability to facilitate Ia IPSPs will be considered in connexion with Table IV and in the Discussion.

High threshold joint afferents

Stimulation of a joint nerve evokes synaptic actions in motoneurones only when the stimulus strength is raised above 2—3×threshold for the lowest threshold fibres (R. M. Eccles and Lundberg 1959 a, Holmqvist and Lundberg 1961, *cf.* however Hongo, Jankowska and Lundberg 1969 concerning low threshold joint effects revealed after supraspinal conditioning stimulation). These effects can thus not be attributed to the low threshold joint afferents connected to Golgi and Ruffini endings (*cf.* Skoglund 1956), but to higher threshold fibres. These high threshold joint afferents are part of the FRA (R. M. Eccles and Lundberg 1959 a). The effect by volleys in high threshold afferents on the transmission in the Ia inhibitory pathway was tested in 26 motoneurones (13 PBSt, 3 DP, 6 Q, 4 G.S.). No facilitation of Ia IPSPs were seen in the 11 motoneurones recorded in low pontine preparations (unanesthetized or under chloralose anesthesia) or unanesthetized spinal animal. In spinal animal with chloralose anesthesia a facilitation was established in 8 of 15 motoneurones as illustrated for a G.S. motoneurone in Fig. 5 and a PBSt motoneurone in Fig. 6 D. It was often possible to abolish the facilitated Ia IPSP by preceding ventral root volleys. Facilitation from high threshold joint afferents and inhibition

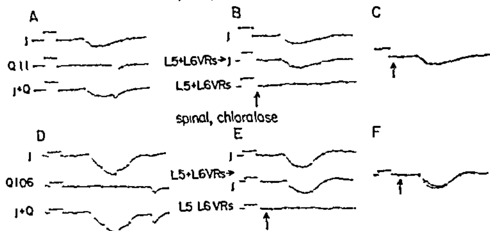


Fig 6 *Comparison of susceptibility to recurrent depression of high threshold joint IPSPs and ability by volleys in the same afferents to facilitate Ia IPSPs* Two PBSt motoneurones were recorded in different preparations—unanesthetized low pontine cat (A—C) and chloralose anesthetized spinal state (D—F) All traces are averaged intracellular responses The records are arranged similar to those in Fig 4 The time of stimulation of ventral roots is marked by arrows The calibration pulses in the beginning of the traces have an amplitude of 1 mV in A—C and 0 5 mV in D—F and a duration of 4 ms The membrane of the motoneurone illustrated in D—F was depolarized by a current of 40 nA

from motor axon collaterals were thus shown to converge onto the same Ia inhibitory interneurones

Fig 6 shows two PBSt motoneurones in which the recurrent depression of the high threshold joint IPSP was compared with the ability by volleys in these afferents to facilitate transmission in the Ia inhibitory pathway A—C were recorded in an unanesthetized low pontine preparation Records B and C show that the joint IPSP was unaffected by the conditioning volley in the L5 and L6 ventral roots and A that a volley in the joint afferents did not facilitate the Ia IPSP The amplitude of the conditioned Ia IPSP was instead profoundly depressed and a close inspection reveals that the sum of the joint IPSP and the conditioned Ia IPSP in fact is less than the amplitude of the unconditioned Ia IPSP alone This indicates that transmission in the Ia inhibitory pathway was inhibited by the joint afferent volley In the other PBSt motoneurone (D—F) which was recorded in a spinal chloralose anesthetized state a small recurrent depression of the joint IPSP (E—F) was revealed as well as a concomitant facilitation of the Ia IPSP from the joint afferents (D) Such a parallelism between susceptibility to recurrent depression of joint IPSPs and ability by volleys in joint afferents to facilitate Ia IPSPs was found in 19 of 22 motoneurones tested In summary the effects evoked from high threshold joint afferents conform with those from high threshold muscle afferents

Cutaneous afferents

The effect by volleys in low threshold cutaneous afferents on transmission in

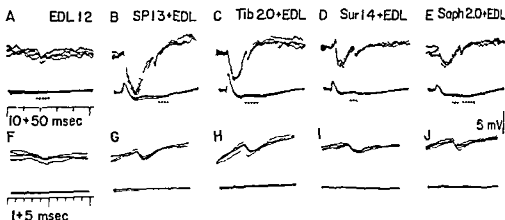


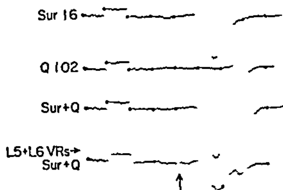
Fig. 7. *Facilitation from low threshold cutaneous afferents of transmission in the Ia inhibitory pathway to an ankle extensor G-S motoneurone.* Upper traces are intracellular potentials. Lower traces are recorded from L7 dorsal root entry zone. Corresponding records in the upper (A—E) and lower (F—J) rows were taken simultaneously at a slow and fast sweep speed respectively. The expanded parts of the slow records are indicated by dashed lines. Time calibration for the slow records in A—E is below A and for the fast records in F—J below F. *Voltage calibration is for the intracellular potentials. Chloralose anesthetized spinal cat.*

Ia inhibitory pathway was tested in 130 motoneurones innervating flexors and extensors at different joints.

Examples of facilitation of Ia IPSPs by volleys in low threshold cutaneous afferents are illustrated in Fig. 7 and 8. The ankle extensor G-S motoneurone illustrated in Fig. 7 was recorded in a spinal cat under chloralose anesthesia. The minute Ia IPSP shown in records in A (and with faster sweep in F) is clearly enhanced by preceding stimulation of SP, Tib, Sur and Saph nerves (B—E, G—J), proving the excitatory convergence from cutaneous afferents and Ia afferents on common Ia inhibitory interneurones.

Ipsilateral cutaneous effects are often not as completely depressed in decerebrate cats as those from high threshold muscle afferents or high threshold joint afferents (R. M. Eccles and Lundberg 1959 b, Holmqvist and Lundberg 1961, *cf.* also Carpenter, Engberg, Funkenstein and Lundberg 1963). Marked early inhibitory actions to extensor motoneurones and facilitatory to flexor motoneurones were often observed in the present experiments, but current passage through the recording microelectrode almost invariably revealed a concomitant IPSP in the latter. A more powerful inhibition of both extensor and flexor motoneurones is, however, seen after a low pontine lesion which releases the inhibitory paths from the high threshold muscle and joint afferents. The records in Fig. 8 are from a knee flexor PBSt motoneurone sampled in an unanesthetized low pontine preparation. The motoneurone was depolarized in order to amplify the IPSP and decrease the EPSP. In this case the initial low threshold EPSP was virtually abolished. The upper traces show the responses to separate stimulation of low threshold cutaneous afferents (Sur 1.6) and of Ia afferents in the Q nerve (Q 1.02), respectively. When stimulated together the Ia IPSP was effectively facilitated. Preceding ventral root stimulation (lower

Fig 8 Convergence of excitation from low threshold cutaneous afferents and inhibition from motor axon collaterals in the Ia inhibitory pathway to a knee flexor PBSt motoneurone All traces are averaged intracellular responses The time of stimulation of L5+L6 ventral roots is marked by the arrow The calibration pulses in the beginning of all traces have an amplitude of 1 mV and duration of 4 ms The motoneuronal membrane was depolarized by a current of 60 nA Unanesthetized low pontine preparation



most trace) almost abolished the facilitated Ia IPSP thus proving the convergence of cutaneous excitation and recurrent inhibition on the same Ia inhibitory interneurones Such a full convergence could almost invariably be established whenever the Ia IPSP was facilitated by volleys in the cutaneous afferents This effect from low threshold cutaneous afferents is in striking contrast to the lack of facilitation from high threshold muscle and joint afferents which on the contrary often inhibited Ia inhibitory transmission in the low pontine state

Table III shows that volleys in low threshold cutaneous afferents facilitated the Ia IPSPs in most PBSt DP and G S motoneurones while such a facilitation was less frequent in Q motoneurones Also in the few cases when a facilitation of a Ia IPSP was seen in the latter it was usually only marginal as compared with those in other species of motoneurones

The ability to facilitate transmission in the Ia inhibitory pathway was tested in all four types of preparation described above but no clearcut differences could be established between them Facilitation of Ia IPSPs was even observed in decerebrate cats without a low pontine lesion (not included in Table III) An inspection of the PBSt material in Table III reveals a tendency for a higher proportion of failures to establish the facilitation in unanesthetized animals as compared with those under chloralose anesthesia (see small print section in the end of Results)

The skin nerves used to condition the test Ia IPSP were those in which volleys could evoke inhibition in the motoneurone recorded In PBSt motoneurones low strength stimulation of the SP nerve often failed to evoke any effect but the early facilitation and the SP nerve was consequently only used rarely to condition test Ia IPSPs in PBSt motoneurones There appeared to be no consistent pattern for the failures to evoke facilitation of Ia IPSPs among the various cutaneous nerve branches in which volleys did evoke inhibition in the motoneurone recorded (*cf* Table III) Qualitative differences in neuronal connexions have earlier been found to fast and slow motor units of the same muscle (Burke Jankowska and Bruggencate 1970) The duration of the afterhyperpolarization (AHP) was therefore measured in several motoneurones (*cf* Eccles Eccles and Lundberg 1958) but it was not possible to establish any correlation between the presence of a facilitation of Ia IPSPs by cutaneous volleys on one hand and the duration of AHP on the other

The facilitation of Ia IPSPs by conditioning stimulation of cutaneous nerves could usually be produced by a stimulus strength of about 1 15—1 2×threshold and was seen to grow up to 2—3×threshold When excitation of the Ia inhibitory interneurones is increased the occlusive effect will increase and may equal or even

TABLE III Facilitation of Ia IPSPs by volleys in ipsilateral low threshold cutaneous afferents The the number of cells tested The type of preparation and the skin nerves tested for spatial brate refer to decerebrate decerebellate cats with a low pontine lesion as described in a spatial facilitation was established of the total number of motoneurones tested in that used It includes a muscle component whose Ia afferents often can evoke Ia IPSPs in

			decerebrate unanesthetized			
			Sur	SP	Saph	Tib
PBSt	(51)	convergence/total	13/22	1/?	1/1	7/10
DP	(18)		2/6	1/2	0/1	—
ABSm	(3)		2/2	—	—	—
Q	(40)		1/13	1/4	—	1/4
G S	(18)		1/1	1/1	1/1	—

exceed the facilitation (ee further below) and it is therefore not possible to exclude that higher threshold fibres also contribute to the excitation of the Ia inhibitory interneurones When the lowest threshold fibres failed to facilitate the test Ia IPSP it was in fact occasionally observed that higher threshold fibres did enh ınce the test (spinal chloralose cats)

The records in Fig 9 illustrate the facilitation of a test Ia IPSP in a Sol motoneurone by conditioning stimulation of the SP nerve with increasing strength Because of a large IPSP from the conditioning volley itself it was not feasible to u e strengths above 1 5 × threshold The time course of the facilitation of the Ia IPSP is own in J Since the onset occurs at a conditioning testing interval of 1 ms it is ulated that in this case no more than 2 interneurones are interposed between the cutaneous primary afferents and the Ia inhibitory interneurone

The recurrent depression of low threshold cutaneous inhibition in motoneurones was often very pronounced and was common in all four types of preparations tested In Fig 10 the recurrent depression of cutaneous inhibition is readily seen even when tested on a monosynaptic reflex A shows the unconditioned PBSt test reflex at two different weep speeds In C and D this test is conditioned by a volley in the Sur nerve The inhibition in C is removed in D when the L5 and L6 ventral roots were stimulated The removal of inhibition in D must be due to a decrease of the cutaneous inhibition since the ventral root stimulation alone did not cause any facilitaton of the PBSt test reflex (B) The graph in E gives the time course of disinhibition obtained with different intervals between the Sur and PBSt incoming volleys (ee further in the legend

Recurrent depres ion of low threshold cutaneous IPSPs was compared in 96 motoneurones (48 PBSt 15 DP 33 Q) with the facilitation of test Ia IPSPs by the same cutaneous volleys Table IV consi ts of 161 comparison since more than one cutaneous nerve was tested in some motoneurones One representative example from the PBSt material is shown in Fig 11 Records in B—C and E—F illustrate that IPSPs evoked by stimulation of the Sur nerve at 1 2 and 1 5 × threshold respectively (upper traces in B and E) were depressed after preceding antidromic volleys in L5 and L6

species of motoneurone investigated are indicated to the left and the figures in parentheses indicate facil tation are given in the upper horizontal rows Note that in the preparations labelled decere Methods The figures presented for each combination show the numbers of motoneurones in which combination (convergence/total) Notice that in case of DP motoneurones the Tib nerve was not these motoneurones thus making the analysis of a possible cutaneous effect more difficult

decerebrate chloralose				spinal unanesthetized				spinal chloralose			
Sur	SP	Saph	Tib	Sur	SP	Saph	Tib	Sur	SP	Saph	Tib
8 9	2/?	—	3/3	1/2	0/1	—	0/1	14/16	2/2	2/2	9 9
3/3	—	—	—	—	—	—	—	5/5	3/3	—	—
—	—	—	—	—	—	—	—	1/1	1/1	—	—
0/5	0/1	—	0/1	0/4	0/1	—	0/5	?/14	2/8	0/4	6/14
2/2	3/3	—	—	2/4	7/8	1/1	1/2	4/4	7/7	—	3/3

ventral roots (middle traces in B and E) The unconditioned and conditioned Sur IPSPs were superimposed in C and F Records in A show the corresponding facilitation of a test Ia IPSP by the same volley which evoked the cutaneous IPSP in B

In cases when low threshold cutaneous IPSPs in PBSt motoneurones were not recurrently depressed there was neither any facilitation of test Ia IPSPs (lower left square) Such a parallelism between susceptibility to recurrent depression and ability to facilitate Ia IPSP was found also in the material for DP and Q motoneurones although the dominance especially in case of Q motoneurones is clearly shifted towards the left lower square *i e* neither any recurrent depression nor any facilitation of test Ia IPSPs

A failure to demonstrate spatial facilitation does not necessarily exclude an excitatory convergence on the Ia inhibitory interneurones—the failure can be caused by a small subliminal fringe which would tend to keep occlusion (also a consequence of convergence) as large as or even larger than facilitation The records in Fig 11 may give some support to this notion The recurrent depression of Sur 1 5 IPSP (E—F) was much larger than that of the Sur 1 2 IPSP If we assume that the recurrently depressed parts of low threshold cutaneous IPSPs in fact represent the fraction which is transmitted via the Ia inhibitory interneurones it follows that far more Ia inhibitory interneurones were fired by the strong than by the weak Sur stimulation The smaller facilitation of the Ia IPSP in case of the stronger Sur stimulation (D) might then be interpreted as an occlusive phenomenon some common Ia inhibitory interneurones may be depressed following the activation from the Sur nerve and fail to transmit from Ia afferents while a larger *facilitation is revealed with weaker Sur stimulus strength (A)* when more interneurones are still in the subliminal fringe We therefore feel that the relatively few occasions (upper left square in Table IV) at which a recurrent depression of low threshold cutaneous IPSPs was not matched by a facilitation of the test Ia IPSPs does not invalidate the main finding of a strong parallelism between susceptibility to recurrent depression and ability to facilitate the test Ia IPSPs

The somewhat less frequent facilitation of Ia IPSPs in PBSt motoneurones from low threshold cutaneous afferents in unanesthetized states as compared with the chloralose anesthetized preparations (Table III) may reflect the greater difficulties to demonstrate the spatial facilitation in preparations with a smaller subliminal fringe Our findings seem to be compatible with this interpretation because the failures to facilitate Ia IPSPs in unanesthetized preparations were often combined with a recurrent depression of the low threshold cutaneous IPSP (upper left square in Table IV all recorded in unanesthetized preparations) It was controlled that the cutaneous volley used at these occasions did not elicit any ventral root discharge (*cf* Discussion)

In a few cases there was a tendency for volleys in cutaneous afferents to facilitate Ia IPSPs although the cutaneous IPSPs were not depressed by the recurrent conditioning volleys This result might be expected if the cutaneous volley only gives a subthreshold excitation of the Ia inhibitory interneurones implying that the entire cutaneous IPSP recorded is due to activation of interneurones other than those mediating the reciprocal Ia inhibition In such cases we al

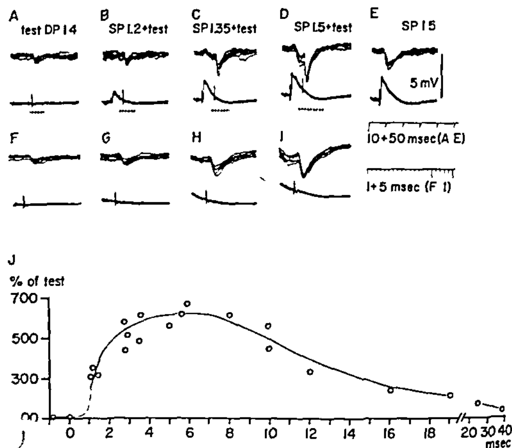


Fig ? Facil tation from low threshold cutaneous afferents of tra ismission in the Ia inhibitory pathway to a Sol motoneurone Upper traces are intracellular potentials Lower traces are recorded from L7 dorsal root entry zone Corresponding records in the upper (A—D) and lower (F– I) rows were taken simultaneously at slow and fast sweep speed respectively The expanded parts of the slow records in A– D are indicated by dashed lines Time calibrations are as indicated below F Voltage calibration is for the intracellular potentials In J the amplitude of the IPSPs conditioned by stimulation of SP 1 5 × thresh expressed in per cents of the unconditioned test response were plotted against the time intervals between the arrival of the conditioning and testing volleys to the spinal cord as measured by a surface electrode at the L7 dorsal root entry zone This motoneurone was record d in an experiment in which the spinal cord wa transected with exception for the ipsilateral dorsal quadrant Cerebellum was acutel removed Chloralose anesthesi

ways raised the strength of stimulation of the cutaneous nerve to learn if the IPSP then was depressed by antidromic volleys This was sometimes the case but a failure (bottom right square for Q motoneur nes in Table IV may be explained if the excitation is still subthreshold for eliciting spikes in the Ia inhibitory interneurones or if the "private IPSP (i e the part of the IPSP which is mediat d via interneur nes oth r than those interposed in the Ia inhibitory pathway) is so domin ting that a pos ible minute d pres ion is overlooked

Considering the experimental difficulties discussed above (cf also in Discussion) the correlation obtained between susceptibility to recurrent depression of low threshold cutaneous IPSP and the ability by volleys in the same afferents to facilitate test Ia IPSPs must be re arded as remarkably good and certainly suggests a causal relationship between the tw phen mena

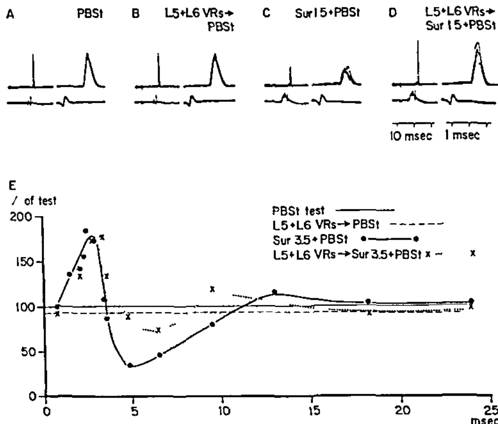


Fig 10 *Recurrent depression of a cutaneous inhibition of a PBSt monosynaptic reflex* Upper traces in records A—D show monosynaptic reflexes recorded in S1 + caudal part of L7 ventral roots lower traces are from the L7 dorsal root entry zone The same responses are shown with two different sweep speeds (*cf* calibrations in D) A series of samples similar to those in A—D are plotted in the graph in E where the amplitudes of monosynaptic test reflexes (ordinate) are plotted against the interval between the Sur incoming volley and the second PBSt volley (abscissa) The interval between the conditioning ventral root volley and the second PBSt volley was kept constant (8 5 ms) The initial facilitation and following inhibition of the PBSt reflex by the conditioning cutaneous volley are evident (points with thick continuous line) The ventral root stimulation produced in fact a small inhibition of the test (thin dashed line) The dashed area represents the decrease of cutaneous inhibition caused by the antidromic conditioning volley Each point or cross represents the average amplitude of about ten test reflexes Unanesthetized low pontine preparation

Discussion

Effects from the FRA on the interneurones mediating the reciprocal Ia inhibition Volleys in ipsilateral FRA in spinal cats evoke inhibition in motoneurones to extensors and excitation in those to flexors (R M Eccles and Lundberg 1959 a) but exceptions with excitation to extensors or inhibition to flexors have been reported (R M Eccles and Lundberg 1959 a Holmqvist and Lundberg 1961 Wilson and Kato 1965) During this study it was usually observed that some inhibition is added to the prevailing excitation in flexor motoneurones The large facilitation of Ia

TABLE IV. Comparison between the susceptibility to recurrent depression of IPSPs from low threshold cutaneous afferents and the ability by volleys in the same afferents to facilitate transmission in the Ia inhibitory pathway. For each individual motoneurone (48 PBSt, 15 DP, 33 Q) the susceptibility to recurrent depression of IPSPs from low threshold afferents of skin nerve was plotted against the ability by volleys in the same afferents to facilitate a test Ia IPSP. In several motoneurones more than one skin nerve was tested resulting in that one motoneurone may be represented by several marks. Different symbols indicate different skin nerves. Notice that the "middle" groups (both at the abscissa and ordinate) mean that it was not possible to decide if the effect was present or absent — no decision

PBSt moton DP moton Q moton

rec depr

no rec depr

no fac of Ia IPSP fac of Ia IPSP

x Sur
O T b
▽ SP
● Saph

A B C

Sur 1.2
Q 1.02
Sur+Q
L5 L6 VRs+ Sur+Q

Sur 1.2
L5 L6 VRs+ Sur
L5+L6 VRs

D E F

Sur 1.5
Q 1.02
Sur Q
L5+L6 VRs+ Sur+Q

Sur 1.5
L5+L6 VRs+ Sur
L5+L6 VRs

FIG. 11. *Facilitation of a test Ia IPSP from cutaneous afferents and recurrent depression of the IPSP evoked from the same cutaneous afferents.* All traces are averaged ... traces ... responses. The records ... arranged similar to those in Fig. 4. The time of stimulation of ventral roots is marked by arrows. The calibration pulse in the beginning of all traces has an amplitude of 1 mV ... The motoneuronal membrane was depolarized by a current of 45 nA ... preparation.

IPSPs in flexor motoneurones in spinal preparations with prevailing FRA inhibition to flexors (*cf* Fig 2) raised the question whether inhibitory FRA actions to motoneurones might be conveyed by interneurones in the Ia inhibitory pathways

Facilitation of Ia IPSPs by volleys in FRA was rather uncommon in the unanesthetized spinal state but appeared regularly after chloralose administration These findings seem to apply to flexor as well as extensor motoneurones The less frequent facilitation of Ia IPSPs by FRA volleys in the unanesthetized state may depend on a weaker FRA excitation of the interposed interneurones in the unanesthetized preparation or reflect that the excitation is obscured by concomitant inhibitory actions Such a concurrent depression cannot however be caused by an interference at primary afferent level since no PAD in Ia afferents is produced by FRA volleys in spinal cats (Eccles Magni and Willis 1962) A depression of Ia IPSPs indicating the existence of an accessory FRA inhibition of the interposed interneurones was rather uncommon in the unanesthetized spinal preparation and when occurring it was often not possible to exclude indirect effects via firing motoneurones and the Renshaw loop The lack of positive evidence for a direct FRA inhibition (*i e* not secondary to firing of motoneurones) in the spinal preparation can hardly be taken as an argument against its existence since the presence of mixed inhibitory and excitatory effects is very difficult to judge with the employed technique In fact FRA IPSPs have been recorded from interneurones supposed to mediate the reciprocal Ia inhibition both in unanesthetized and chloralose anesthetized spinal cat (Hultborn *et al* 1971 b Hultborn and Santini to be published)

A study of how Ia IPSPs in motoneurones are influenced when the Ia inhibitory interneurones receive a mixture of EPSPs and IPSPs will usually disclose the dominant action The outcome may however depend on the size of the applied test IPSP as is illustrated by the following example Let us assume that a conditioning volley evokes mixed excitation and inhibition in the Ia inhibitory interneurones and that the net effect is just excitatory in half of the interneurones and just inhibitory in the rest If the test Ia IPSP is small—*i e* only a few interneurones are fired while many are in the subliminal fringe—the conditioning volley is likely to facilitate the test IPSP because the inhibition could stop the firing maximally in half of the few interneurones discharged by the test shock while the facilitation has the chance to fire half of the large population in the subliminal fringe If the test IPSP on the other hand is large the above argument can be utilized to show that the conditioning volley gives a depression of the test IPSP Since we have been mainly interested in the facilitatory action on the Ia inhibitory interneurones we have used weak test IPSPs which were appropriate for revealing facilitatory actions An accessory FRA inhibition of these interneurones may thus have been overlooked

In decerebrate cats with a low pontine lesion FRA volleys evoke inhibition in both flexor and extensor motoneurones (Holmqvist and Lundberg 1961) but volleys in high threshold muscle or joint afferents did not facilitate Ia inhibitory transmission in this preparation (unanesthetized or under chloralose anesthesia *cf* Table I Fig 4 and 6) On the contrary volleys in the FRA often evoked a depression of Ia IPSPs (Fig 4 and 6) It was possible to exclude that this depression was caused by conductance or potential changes in the motoneurone recorded The reduction of the Ia IPSPs can neither be explained by presynaptic inhibition of transmission from Ia afferents since FRA volleys in decerebrate cats (also in those with low pontine lesions) does not evoke DRPs (Carpenter *et al* 1963)

During failing or incomplete decerebrate control of reflexes to primary afferents volleys in FRA may produce late and longlasting DRPs resembling those which are evoked in spinal cats after injection of monoamine precursors (Andén *et al.* 1966 b). The latter DRPs are in fact caused by a PAD in Ia afferents but the central latency is about 100 ms (Andén *et al.* 1966 b). Since the conditioning testing intervals in this study were kept below 30 ms it is excluded that a PAD in Ia afferents—even if present—could be responsible for the obtained depression of Ia IPSPs.

Since the depression of Ia IPSPs by volleys in FRA cannot be ascribed to interference at a motoneuronal or primary afferent level it must be caused by inhibition of the interposed interneurone. It was excluded that this inhibition was secondary to firing of motoneurones and thus mediated via the Renshaw loop. This FRA inhibition may be either post- or presynaptic but it is recalled that Hultborn *et al.* (1971 b) sometimes found postsynaptic inhibition from FRA on direct recording from interneurones supposed to mediate the reciprocal Ia inhibition to motoneurones. Taken together our findings suggest that the Ia inhibitory interneurones—like the motoneurones (Holmqvist and Lundberg 1961)—receive FRA inhibition in the decerebrate preparations with a low pontine lesion.

These findings suggest the existence of a descending inhibitory control of the pathway which mediates the facilitatory action from the FRA to the Ia inhibitory interneurones. The question may be raised why the low pontine lesion known to release transmission in other inhibitory pathways from the FRA does not release the pathway utilizing the Ia inhibitory interneurones. The simplest explanation may be that the FRA excitation of the Ia inhibitory interneurones in fact constitutes a collateral effect to the FRA excitation of the motoneurones which is not released by the low pontine lesion. Facilitation of Ia IPSPs by FRA volleys would then be expected only when the descending inhibitory control of the excitatory FRA pathway to motoneurones is broken, *e.g.* after spinalization (Holmqvist and Lundberg 1961). If so, it follows that the FRA connections to motoneurones and Ia inhibitory interneurones have a principally similar organization. The "private" FRA inhibition (not mediated via the Ia inhibitory interneurones) seen in the low pontine preparation would then be essentially different from the FRA inhibition mediated by the Ia inhibitory interneurones, the latter in a sense being more closely tied to the FRA excitation of motoneurones.

The observation that chloralose administration ensures a much more regular facilitation of Ia IPSPs than in the spinal unanesthetized state could be interpreted so that the balance between FRA EPSPs and IPSPs in the Ia inhibitory interneurones is shifted and stabilized in favour of the former. There is at present no indication of how this might be achieved but we do not find it necessary to postulate any qualitative changes like *e.g.* opening of a previously closed excitatory pathway from FRA to the Ia inhibitory interneurones.

Effects from cutaneous afferents on the interneurones in the Ia inhibitory pathway. Low threshold as well as high threshold cutaneous afferents are part of the FRA (R. M. Eccles and Lundberg 1959 a), but there is now a bulk of evidence showing that the low threshold afferents in addition have separate reflex pathways to moto-

neurones (Hagbarth 1952 Engberg 1964 Hongo *et al* 1969 Baldissera Bruggencate and Lundberg 1971 *cf* also Hongo Jankowska and Lundberg 1966) Our present finding that volleys in ipsilateral low threshold cutaneous fibres facilitate the Ia IPSPs in decerebrate cats (with or without low pontine lesion) although there is no corresponding facilitation from high threshold muscle and joint afferents would likewise be explicable if we assume that the effect is exerted by separate reflex pathways from low threshold cutaneous afferents

The occasional finding that the central latency of the facilitation of a test Ia IPSP may be as brief as 1 ms (Fig 9) may be compatible with a disynaptic—or at least not more than trisynaptic—linkage between cutaneous primary afferents and Ia inhibitory interneurones Direct recording from interneurones supposed to mediate the reciprocal Ia inhibition (Hultborn and Santini to be published) indicates however that the minimum linkage is trisynaptic

The functional significance of the convergence from various segmental afferents on the interneurones in the Ia inhibitory pathway will be discussed elsewhere (Hultborn 1972)

Are recurrently depressed parts of IPSPs from the FRA and separate cutaneous pathways mediated by Ia inhibitory interneurones?

The mere finding of a facilitation from the FRA or from low threshold cutaneous afferents of transmission in the Ia inhibitory pathway does not prove that volleys in these afferents are capable of evoking IPSPs in motoneurones by discharging the Ia inhibitory interneurones Direct recording from interneurones supposed to mediate the reciprocal Ia inhibition has however shown that such afferent volleys in fact may cause a vigorous firing of them (Hultborn *et al* 1971 b Hultborn and Santini to be published) The observations that Ia IPSPs which have been facilitated by volleys in cutaneous afferents or in the FRA can be abolished by preceding volleys in ventral roots show that excitation from them and inhibition from motor axon collaterals converge onto the same Ia inhibitory interneurones It can therefore be concluded that volleys in these segmental afferents *can* evoke inhibition in motoneurones via Ia inhibitory interneurones and that this inhibition *can* be depressed by antidromic volleys in motor axons Does the lack of recurrent depression of a segmental IPSP then exclude that it is mediated by the Ia inhibitory interneurones? An answer in the affirmative would require that *all* the interneurones mediating reciprocal Ia inhibition are susceptive to recurrent inhibition This has not been proved but there is on the other hand no evidence for Ia inhibitory interneurones lacking recurrent inhibition (Hultborn *et al* 1971 b)

The present systematic study of recurrent depression of IPSP from various afferents and the ability by volleys in the same afferents to facilitate Ia IPSPs has revealed a remarkably strong positive correlation between these events This correlation means not only that afferent volleys which facilitate Ia IPSPs also can evoke IPSPs susceptive to recurrent depression (*cf* the conclusion above) but conversely also that a recurrent depression of an IPSP from a given group of afferents is seen

only (with few exceptions) when a volley in these afferents cause a facilitation of a test Ia IPSP. This supports the idea that recurrent inhibition of interneurones in ipsilateral afferent pathways to motoneurones is restricted to those mediating the reciprocal Ia inhibition.

It cannot be entirely excluded that the recurrently depressed parts of various segmental IPSPs have been mediated by Renshaw cells since recent investigations have revealed the existence of a mutual inhibition between the Renshaw cells themselves (Ryall 1970, Ryall, Piercey and Polosa 1971). However, even if a part of the segmental IPSPs were conveyed by Renshaw cells it is unlikely that even that part would be effectively depressed by conditioning ventral root volleys since the inhibitory interaction between Renshaw cells seems to be very weak (Hultborn, Jankowska, Lindström and Roberts 1971, see their Fig. 6).

Afferent volleys may excite Renshaw cells—and thus evoke IPSPs in motoneurones—secondary to firing of motoneurones. We have therefore strived to use afferent nerve volleys which did not elicit any discharge in ventral roots but an ensuing firing of motoneurones could often not be avoided in the spinal preparation (see Results). It is of importance to notice that the strict correlation between susceptibility to recurrent depression of various IPSPs and the ability to facilitate Ia IPSPs by volleys in the same afferents (Table II and IV) was independent of whether a ventral root discharge was evoked. It was also especially remarked in the Result section that no firing of motoneurones was elicited by the nerve volleys which evoked the IPSPs susceptive to recurrent depression but no corresponding facilitation of test Ia IPSPs (exceptions in upper left squares in Table II and IV).

It has been postulated that ipsilateral segmental afferents may fire Renshaw cells also directly (i.e. not via motoneurones) (Eccles, Fatt and Koketsu 1954, Frank and Fuortes 1956, Curtis, Phillis and Watkins 1961). It shall, however, be recalled that the prevailing "direct" effects on Renshaw cells by impulses in spinal afferents consist of an inhibition which is often described as powerful (Wilson, Talbot and Kato 1964, Wilson 1966). It thus seems unlikely that IPSPs in motoneurones from ipsilateral segmental afferents are evoked by "direct" activation of Renshaw cells.

In summary the above arguments indicate that recurrent depression of ipsilateral segmental IPSPs (which are not secondary to firing of motoneurones) reflects that—and also roughly to which extent—they are mediated by the Ia inhibitory interneurones. It has appeared that FRA IPSPs in motoneurones in the spinal chloralose anesthetized state are partly conveyed by the interneurones in the Ia inhibitory pathway and more so in case of flexor motoneurones than of extensor motoneurones. In the low pontine preparation the FRA inhibition seems to be mediated entirely by private FRA interneurones (which are not shared with Ia afferents). Inhibition of motoneurones exerted by separate reflex pathways from low threshold cutaneous afferents was also to a varying degree mediated by the Ia inhibitory interneurones.

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Shunting by Diffusion of Inert Gas in Skeletal Muscle

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Abstract

SEJRSEN P and K H TONNESEN *Shunting by diffusion of inert gas in skeletal muscle* Acta physiol scand 1972 *86* 82—91

The isolated cat gastrocnemius preparation was used to study shunting by diffusion of ^{133}Xenon from arterial to venous blood ^{133}Xenon in saline ^{51}Chromium labelled erythrocytes and T1824 albumin were mixed and injected as a bolus into a side branch of the femoral artery The muscle was autoperfused and stimulated electrically Gas tight samples were taken every 0 7 to 1 0 s with a *special technique developed for this purpose The effluent blood* from the muscle was collected in a 1 5 m long glass tube (i d 2 to 3 mm) and from a side branch at the inflow end of the tube mercury drops were led into the tube dividing the blood stream into samples The inflow was stopped after [illegible]0 seconds and the contents of the two isotopes in the samples were measured by collimated counting externally over the tube The T1824 analysis were performed spectrophotometrically ^{133}Xenon appeared in the venous samples before the intravascular reference tracers indicating that ^{133}Xenon was shunted by diffusion from arterial to venous blood The fraction of ^{133}Xenon shunted by diffusion was estimated to about 11 per cent

In 1929 August Krogh suggested that exchange of substances between blood and tissue takes place not only through the walls of capillaries but also through the walls of small venules Studies on skeletal muscle with highly diffusible molecules have supported this suggestion showing that also larger venules and veins exchange small lipophilic molecules with the tissue and with the blood in the arterial vessels (Aukland *et al* 1967 Sejrsen and Tonnesen 1968 Duling and Berne 1970) This exchange is denoted *shunting by diffusion* and is *known from heat exchange* (Bazett *et al* 1948 Roed and Aukland 1969) The purpose of the present study is directly to demonstrate shunting by diffusion between arterial and venous vessels of highly diffusible molecules by comparing the simultaneous passage of a diffusible tracer to that of an intravascular one Both precession of the highly diffusible molecule and an extraction lower than expected indicate shunting by diffusion The theoretical consideration on extraction are discussed

Experimental

The isolated gastrocnemius muscle in cats was used as described previously (Tonnesen and Sejrsen 1967) In this preparation all visible fat was removed To obtain steady blood pressure and hemoglobin concentration the cats were given donor blood

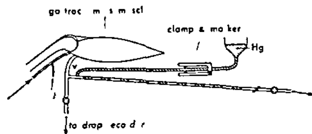


Fig 1 The experimental set up The gastrocnemius muscle is isolated except from the femoral bone and the vessels The Achilles tendon is fastened to a stand The T tube connected to the vein conducts the blood either to the drop recorder or to a long *slightly* declining glass tube From the reservoir mercury drops can be introduced into the glass tube close to the vein by opening the clamp The tracer is introduced through the thin catheter in a side branch of the femoral artery

A thin polyethylene catheter (i d 0 4 mm) was inserted into a side branch of the distal part of the femoral artery To obtain rapid and gas tight samples from venous blood a T tube was inserted into the femoral vein clo e to the muscle (Fig 1) The blood could flow from the muscle to a central vein via a photoelectric drop-counter A third branch was connected with a second T tube leading to a 1 5 m long glass tube (i d 2 to 3 mm) To compensate for the flow resistance in the tube the free end of the glass tube was 10—15 cm below the connection with the T tube The third branch of the second T tube was connected with a latex tube leading to a reservoir filled with mercury The surface of mercury in the reservoir was about 30 to 40 mm above the level of the second T tube The latex tube could be closed by a clamp The position of the clamp was registered by a marker on a 4-channel Beckman dynograph type R on which the *arterial blood pressure, the drop-counter flo*w before the injection and the duration of the samples were recorded

Before the injection of the tracers 3 to 4 blanks were taken each divided with a drop of mercury A tracer bolus of about 0 1 ml containing a mixture of ^{51}Chromium labelled erythrocytes (about 40 μCi ^{51}Chromium) and ^{133}Xenon (about 40 μ Ci) was injected through the thin catheter into the side branch of the femoral artery in 1 s The catheter was only 30 to 40 mm long In 1 expt the tracer bolus al o contained $T_{1\ 21}$ albumin (about 0 ? mg T_{1524})

Simultaneously with the start of the bolus injection the tube leading to the drop-counter was closed and the passage into the long glass tube was opened A drop of mercury was placed in front of the inflowing blood Every 0 7 to 1 0 s a drop of mercury was introduced into the glass tube separating the blood column into samples The blood samples in the tube had a length of 14 to ?6 mm and the mercury drops a length of 18 to 36 mm When 22—27 samples were taken the inflow into the glass tube was stopped and the passage to the drop-*counter was reestablished In* one case two experiments were made with *the same preparation* In the time between the two experiments recirculation was avoided and the wasted blood was replaced by donor blood

Standa ds vere prepared from the original tracer mixture by mixing the tracers in a 1 ml tuberculin syringe and transferring 0 1 ml of the mixture into a 10 ml syringe filled with saline by injection through a rubber stopper The dilution factor was measured by weighing The diluted tracer mixture was transferred into a cut section of the long glass tube and separated *by mercury drops* The standard samples were made so that the range of their length corresponded to the range of the venous samples The activity in the venou samples was corrected to a standard length

The radioactivity was counted by a scintillation crystal (Na I (Tl)) The output from the detector was divided into two amplifier analyzers (Philips PW 4?80) one of which integrated a suitable window around the 81 kev γ-ene gy of ^{133}Xenon and the other integrated around the 3?3 kev γ energy of ^{51}Chromium The counts ere registered b two s alers with printout. *The Compton scatter from* ^{51}*Chromium* was registered in the 81 kev channel by 1? 7 per cent. The ^{133}Xenon counts were corrected accordingly The γ-energy of ^{133}Xenon did not influence the count rate in the 323 kev channel The scintillation probe was sl lded by lead bri ks and the background count rate was kept about 9 cpm (range 6 4 to 15 0 pm) in the 81 ke channel and about 9 cpm (range 7 2 to 11 3 cpm) in the 3 3 ke channel The front surface of the probe was co ered with a 12 mm thick shield of lead w th a nt ally placed hol which was 17 mm in diameter The sampling glass tube was placed cent ll in f ont of the hole and covered by *two lead br cks By means of* this a constant counting g om etry and a lo back ground acti ity were obtained

The 24 h decay for ^{133}Xenon and ^{51}Chromium could be accounted for by the physical decay for all samples. The background and the activity in the venous blood samples containing significant amounts of activity were counted with an accuracy of at least 10 per cent. T 1824 was measured by spectrophotometry.

To obtain samples of about 100 μl every second it was necessary to have a sufficiently high blood flow. Therefore the cut sciatic nerve was stimulated in all experiments by silver electrodes using a S4 Grass stimulator. The frequency was 2 to 3/s, the voltage 6 to 8 V and the duration 1 ms. The output from the photoelectric drop counter was recorded. The drop-counter was calibrated by cylinder glass and stop watch. The blood flow was determined immediately before the tracer injection. After the measurement of the radioactivity the glass tube was cut into pieces to collect the samples for T 1824 analysis. In all samples at least 25 μl of plasma could be obtained. Blank plasma was added up to a volume of 100 μl and further 200 μl of saline. The readings were made on a Beckman DU spectrophotometer at 620 nm using semi micro cuvettes. A calibration curve was made in each experiment from the standard solution in the 10 ml syringe.

Theoretical considerations

The partition coefficient λ is defined as the volume of blood which has the same solute capacity as one gram of the tissue (Kety 1951). If two tracers with widely different partition coefficients are injected simultaneously into the artery of the tissue and the concentrations are measured in the venous effluent blood, the mean transit time for the two tracers is proportional to their respective λ values, i.e. to their relative volumes of distribution. If the tracer is albumin bound, the volume of distribution equals the plasma volume. If the tracer is freely diffusible in tissue as ^{133}Xenon, the volume of distribution includes the whole tissue mass but λ or the relative volume of distribution is only about 0.7 ml/g. Thus the volume of distribution (V) is for ^{133}Xenon an imaginary volume which can be obtained by multiplying the λ value by the weight (W) of the tissue: $V = \lambda \cdot W$. The ratio between the volumes of distribution for the two tracers is equal to the ratio of the λ values.

The partition coefficient λ is also defined as the ratio between the solubility of the gas in tissue and in blood:

$$\lambda = \frac{\text{concentration in tissue at equilibrium}}{\text{concentration in blood at equilibrium}} \ (\text{ml/g}) \qquad (1)$$

This ratio is expressed as ml/g because the blood was measured in ml and the tissue was weighed in g (Kety 1951). The amount of tracer within the blood in the vessel of the tissue is included in the tissue concentration. This volume is a fraction of the volume of the washout system which is defined as the volume between the input site and the sampling site. The partition coefficient λ for ^{133}Xenon was calculated from the hematocrit values by using the results of a previous study (Tonnesen and Sejrsen 1967) where λ was measured at different hematocrit values.

The intravascular volume of the muscle including in- and outflow catheters was calculated in each experiment by use of directly measured blood flow and the mean transit time for ^{51}Chromium labelled erythrocytes. Using these λ values, the mean transit time $\bar{t}$ for ^{133}Xenon is about 14 times larger than that of ^{51}Chromium labelled erythrocytes (in the present experimental set up).

The concentration of the tracers in the samples, expressed relative to the concentration in the injectate, should then be about 14 times smaller for ^{133}Xenon than for ^{51}Chromium labelled erythrocytes.

The γ-emission is 9 per cent of the disintegrations for ^{51}Chromium and 36 per cent for ^{133}Xenon respectively. By combining the percentage of γ-emissions and the volumes of distribution the likelihood of detecting ^{51}Chromium can be estimated to be about 3.5 times greater than the likelihood of detecting ^{133}Xenon if the same amount of the two tracers (in μCi) is used.

If some ^{133}Xenon appears in the venous samples before the ^{51}Chromium labelled erythrocytes and if the relative concentration of ^{133}Xenon in the increasing phase and around the peak concentration is higher than the relative concentration of the intravascular tracers, the ^{133}Xenon must have [illegible] the blood stream by diffusion, i.e. extravascular shunting by diffusion.

Extraction

The extraction of a tracer permeating the capillary wall can be estimated by comparison with a tracer staying inside the vessels (Crone 1963). However it is only valid to calculate the capillary extraction if the ratio of permeable tracer to intravascular tracer is the same in the

TABLE I Muscle weight and edema directly measured blood flow and hematocrit values

Expt No	Muscle weight g	Muscle edema in	Directly measured blood flow		Hematocrit in %
			µl/sec	ml/100 g min	
1	19 9	1 3	58	17 6	28
2	35 2	11.2	206	35 5	33
3	35 2	11 2	217	36 9	21
4	34 6	3 0	175	30 3	48

injectate and in the capillaries If some Xenon molecules diffuse from the arterial vessels into the surrounding tissue and possibly into the venous vessels before the bolus reaches the capillaries then the ratio of permeable tracer to intravascular tracer in the capillaries is less than assumed and the calculated capillary extraction becomes erroneously low To calculate a possible a v loss of the gas tracer along the vessels the extraction was calculated from measured values and compared to the extraction which could be expected

The fraction of ^{133}*Xenon which leaves the blood during the passage through the exchange vessels i e* the extraction E in per cent can be calculated according to Crone (1963) as

$$E = \left[1 - \frac{\text{relative concentration of } ^{133}\text{Xenon in sample}}{\text{relative concentration of intravascular tracer in sample}}\right] 100 \qquad (2)$$

The concept of extraction after intra arterial bolus injection of a permeable tracer (Chinard 1954) is only valid when compared to an intravascular tracer in the short time interval of the increase and the first part of the decrease of the concentration time curve for the intra vascular tracer Hereafter backdiffusion from the tissue into the blood takes place and the extraction value decreases (Crone 1963) If a plateau is present in the extraction curve as seen in Fig 2 the bottom diagram it is generally taken as an expression of insignificant influence of backdiffusion in the phase of the plateau

The expected extraction for ^{133}Xenon in skeletal muscle may be calculated from the capillary blood volume and the λ value for ^{133}Xenon by assuming diffusion equilibrium in the tissue capillary cylinder' The capillary blood volume is less than one per cent of the tissue volume and using a λ value of 0 66 corresponding to a hematocrit of 33 per cent (*Tønnesen and Sejrsen* 1967) then the amount of ^{133}Xenon in the outflowing blood would be less than 1 5 per cent of the concentration in the injectate i e an extraction exceeding 98 per cent If the extraction is substantially less than 98 per cent four possibilities must be considered (1) It may be due to relatively large diffusion distances in the tissue (2) a permeability resistance in the capillary wall for ^{133}Xenon (3) true arteriovenous blood shunts and/or (4) shunting by diffusion. The three first *mentioned possibilities are eliminated as main factors* by the experimental finding of blood flow limited washout of ^{133}Xenon (Sejrsen and Tønnesen 1968) The fourth possibility which is the most likely shall here be given a few comments The amounts of ^{133}Xenon which appears in the venous samples before the ^{51}Chromium labelled erythrocytes cannot give a quantitative measure of the shunt fraction—*or the loss of* ^{133}Xenon from the arterial vessels to the tissue before the capillaries because a *further redistribution—reshunting—will* occur However the extraction value gives a fair estimate of the net loss of ^{133}Xenon from the arterial to the venous vessels when the observed extraction is compared to the expected extraction

Results

4 exps were made on 3 cats The muscle weight the edema the directly measured blood flow and the hematocrit values are given in Table I The muscles were severed at the end of the experiments The contralateral *gastrocnemius muscle* was removed By comparing the weights of the 2 muscles it was estimated that little edema had occurred in the muscle used in the experiment Therefore no correction for the edema was used in the calculation of the blood flow

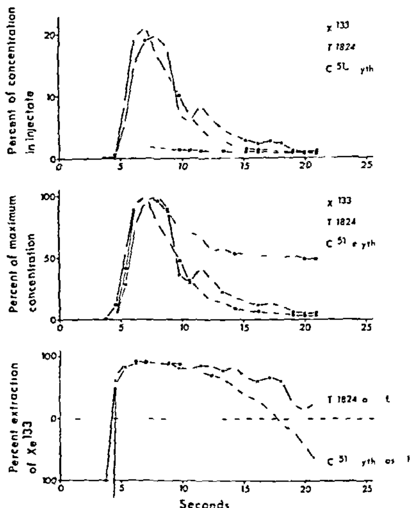


Fig. 2. Venous concentration as a function of time in per cent of concentration in injectate (above) and in per cent of maximum concentration (middle). Below: Extractions of 133 Xenon calculated from equation (2).

The concentration curves for samples of the effluent blood from exp. no. 4 are seen in Fig. 2. In the upper graph the concentrations are given in per cent of the concentration in the injectate. The relative concentrations of 133 Xenon in the samples are about one tenth of the relative concentrations of the intravascular tracers at maximum due to the larger volume of distribution for 133 Xenon. In this graph it is possible to see that the relative 133 Xenon concentration in the first sample is larger than those of 51 Chromium and T 1824. The 51 Chromium labelled erythrocytes were found to have slightly faster tran it times than T 1824, which is in accordance to the findings of Groom (1968) at similar hematocrit values.

In the middle graph the concentrations are given in per cent of maximum concentration for each tracer. The 133 Xenon concentration was larger in each sample

TABLE II Counts per minute in injectate relative volumes of distribution λ and maximum counts per minute in samples for ¹ Xenon and Chromium labelled erythrocytes

Expt no	Injectate: Xe cpm	Injectate: ^{51}Cr eryth cpm	Relative volume of distribution λ: λ Xe	λ ^{51}Cr eryth	$\dfrac{\lambda\ Xe}{\lambda\ eryth}$	Expected ratio: $\dfrac{^{1}Xe\ cpm/Cr\text{-}eryth\ cpm}{\lambda\ Xe/\lambda\ Cr\text{-}eryth}$	Measured max count rate in samples: ^{13}Xe cpm	^{51}Cr eryth cpm	$\dfrac{^{1}Xe}{Cr\ eryth}$
1	148 000	45 500	0 70	0 047	15	0 22	8474	19 080	0 44
2	234 000	74 000	0 66	0 061	11	0.29	1669	2 58	0 6
3	213 000	18 600	0 77	0 044	18	0 64	2719	2 474	1 10
4	101 700	28 700	0 51	0 035	15	0 24	1782	6 043	0 30

The relative volume of distribution λ for ¹ Xenon is taken from the values given in Tonnesen and Sejrsen (1967) taking the hematocrit into account The volume of distribution for Cr labelled erythrocytes is calculated from their mean transit time through the muscle and sampling catheter and the directly measured blood flow

relative to the standard until the maximum of the curve The first sample which contained detectable tracer contained a small but significant amount of ^{133}Xenon but no significant amount of the intravascular tracers. The calculated extractions according to equation (2) are seen in the bottom diagram in Fig 2

The count figures for ^{133}Xenon and ^{51}Chromium labelled erythrocytes and their respective λ values are seen in Table II From these figures the theoretical possibility

TABLE III Count figures for Xenon and Chromium labelled erythrocytes in the initial samples and the corresponding extraction values after correction for background and Compton scatter

Expt no	Sample no	Count figures and standard deviation* in cpm: ¹ Xe		¹Cr eryth		Extraction E in
1	4	60 8 SD 2 1	$p \ll 0\ 0001$	9 8 SD 0 9	$p \ll 0\ 0001$	− 90
	5	553 SD 7 4		29 SD 3 9		42
	6	1481 SD 21		1638 SD 13		72
2	5	16 2 SD 0 9	$p \ll 0\ 0001$	1 1 SD 0 7	$p < 0\ 12$	−360
	6	129 SD 2 8		73 6 SD 2 1		55
	7	463 SD 10		503 SD 10		71
3	5	5 1 SD 0 4	$p \ll 0\ 0001$	1 2 SD 0 4	$p < 0\ 003$	63
	6	216 SD 5 0		250 SD 4 9		9? 5
4	5	7 1 SD 0 7	$p \ll 0\ 0001$	1 0 SD 0 7	$p < 0\ 16$	−100
	6	210 SD 5 0		181 SD 4 4		68
	7	990 SD 11		2493 SD 16		89

* In the calculation of the standard deviation of the count figures the following ariances are taken into account The count figure of the isotope in question th count figure due to Compton scatter from the other isotope and the b ckground in both channels The p-values ndicate the probab lity of the count figu e to be equal to the background

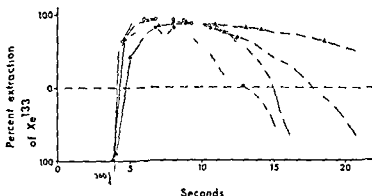


Fig. 3. Calculated extractions of ^{133}Xenon from all 4 expts. Exp. no. 1 is indicated by open triangles, no. 2 by closed circles, no. 3 by open circles and no. 4 by closed triangles respectively.

of detecting the 2 tracers can be calculated. The relation is expressed as the expected ratio. The possibilities of detecting ^{51}Chromium are from about 2 to 5 times as good as for detecting ^{133}Xenon. These considerations are of importance to discriminate the count figures of the tracers in the first samples from those of the background activity. The count figures for the first samples which contain tracer are given in Table III. The figures are corrected for background and Compton scatter and they have been subjected to statistical analysis. In sample no. 5 in exp. no. 2 and 4 a statistically significant difference was found between the background and the count figures for ^{133}Xenon but not for ^{51}Chromium labelled erythrocytes.

In addition the negative and increasing extraction values initially indicate the appearance of ^{133}Xenon before the intravascular tracers as mentioned in theoretical consideration. In the samples before maximum of the curve the relative ^{133}Xenon concentration was found to be higher than the relative ^{51}Chromium concentration when the z values for the two tracers are considered. This indicates shorter transit times for a fraction of the ^{133}Xenon bolus compared to the labelled erythrocytes. The extraction values for the total period of the study appear in figure 3 for all four experiments.

The maximum extraction level in the four experiments was 86, 80, 92 and 91 per cent. The mean value 87 (S.D. 6 per cent, S.E. 3 per cent) is significantly lower than the theoretical value of 98 per cent which was expected in case of equilibrium and without shunting by diffusion.

Discussion

After intra-arterial bolus injection close to the muscle of a mixture of ^{133}Xenon and an intravascular tracer, ^{133}Xenon appeared in the venous blood samples before the intravascular tracer. This can only occur if ^{133}Xenon short circuits the intravascular route, i.e. shunting by diffusion.

The subsequent samples before maximum contained relatively more ^{133}Xenon compared to intravascular tracer than could be expected from the λ values of the 2 tracers This emphasizes the above mentioned finding of shunting by diffusion from the blood in the arterial vessels to blood in the venous vessels

When ^{133}Xenon gas is lost from the arterial blood into the venous blood the ratio of the concentrations of ^{133}Xenon and of ^{51}Chromium in the capillary must be lower than that of the injectate In the present experiments the extraction was found to be less than theoretically expected in all experiments indicating loss of ^{133}Xenon from the arterial to the venous blood The experiments give only a figure of the net fraction of gas which is shunted by diffusion As it was demonstrated that diffusion equilibrium exists for ^{133}Xenon in the capillaries (Sejrsen and Tonnesen 1968) then an extraction of more than 98 per cent is to be expected However the average extraction was only 87 per cent This indicates a shunt fraction of about 11 per cent

In the present experiments relatively high values of blood flow were used At lower flows a higher shunt fraction must be expected This is in accordance with the findings of Bassingthwaighte and Yipintsoi (1970) in cardiac muscle They found indications of shunting by diffusion of ^{133}Xenon and other tracers especially when the coronary blood flow was relatively low Furthermore the counter-current exchange depends on the diffusion coefficient Therefore the shunt fraction on this basis should be larger for smaller molecules such as oxygen

A study of periarteriolar oxygen tensions by means of microelectrodes (Duling and Berne 1970) suggests a loss of oxygen along the arterial tree from 20 to 50 per cent These results which are in accordance to ours if the difference in experimental procedure the lower blood flow and the smaller molecular size are considered yield also support to the previous suggestions of exchange of gases through the walls of vessels larger than capillaries (Krogh 1929 Aukland *et al* 1967 Sejrsen and Tonnesen 1968 Sejrsen 1967 1969)

Aukland *et al* (1967) registered the hydrogen desaturation curve in the effluent blood from skeletal muscle after complete saturation In these experiments the initial desaturation phase measured in the venous outflow was faster than could be explained by the blood flow This might be due to shunting by diffusion of hydrogen gas or to a true blood shunt The last possibility can be excluded both from Hammersen s anatomical studies of the vasculature in skeletal muscle where no true arteriovenous shunts were found (Hammersen 1970) and from our previous studies (Sejrsen and Tønnesen 1968 Tonnesen and Sejrsen 1970) where the externally registered initial desaturation rate after complete saturation with ^{133}Xenon equalled the directly measured blood flow If a true blood shunt of significant size existed the directly measured blood flow would exceed the blood flow calculated from the ^{133}Xenon washout rate in the last mentioned studies In these studies where isolated cat gastrocnemius muscle was used saturation was made both intraarterially and locally As the initial washout rate agreed with the directly measured blood flow a high degree of diffusion equilibrium between tissue and blood is liable As the experiments with local labelling also demonstrated that the directly measured blood

flow equalled the blood flow calculated from the initial washout rate the slower washout rate in the later part of these curves cannot be explained by inhomogeneous distribution of the blood flow The small labelled areas were of such a size (from 0 05 to 1 ml) that diffusion gradients large enough to explain the bended shape of the curve simply could not exist during the washout process because this would imply very large concentration gradients within the small labelled area Therefore we suggested (Sejrsen and Tonnesen 1968) the bended shape of the curve to be explained predominantly as shunting of ^{133}Xenon by diffusion This hypothesis explains both the initial rapid phase and the slow tail part of the curves after bolus injection as an initial a v shunting and a later v a shunting The shape of the washout curves fom small areas labelled locally with ^{133}Xenon (Sejrsen and Tonnesen 1968 Tonnesen and Sejrsen 1970) suggests that the diffusional gas transfer occurs from blood in venules to blood in arterioles This is supported by the close relations between smaller arterioles and venules (Hammersen 1964) Also for larger vessels shunting by diffusion may take place in skeletal muscle as the arterious and venous vessels run concomitantly through the tissue As the surface to volume ratio decreases for larger vessels the possibilities of shunting by diffusion also decreases

At least in three fields of physiology shunting by diffusion is of importance (1) interpretation of washout curves of radioactive gases used in blood flow measurements (2) uptake and distribution of anesthetic agents and (3) oxygen and carbon dioxide transport It is suggested that shunting by diffusion of highly diffusible molecules is of significance in other tissues

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From the Department of Physiology University of Goteborg Sweden

The Role of Muscle Stiffness in Meeting the Changing Postural and Locomotor Requirements for Force Development by the Ankle Extensors

By

S Grillner

Received 11 February 1972

Abstract

Grillner S *The role of muscle stiffness in meeting the changing postural and locomotor requirements for force development by the ankle extensors* Acta physiol scand 1972 86 92—108

The relationship between the length and stiffness (force/length change) of active muscle and the rates at which motor units are activated by distributed stimulation of divided ventral rootlets supplying the muscle has been explored in cat soleus and lateral gastrocnemius At stimulus rates equivalent to those used in locomotion (15—55 p/s) peak muscle stiffness is at muscle lengths also used in locomotion At lower rates of stimulation equivalent to those developed in the tonic stretch reflex of the decerebrate cat peak muscle stiffness occurs at larger muscle lengths but these lengths are within the range of those used for investigation of the reflex These experimental results were then compared to the sets of theoretically derived results concerning a) the amount of force needed from the ankle extensors to counteract a load applied to the hindlimb along a vertical axis when ankle and metatarsophalangeal angles are changed relative to the mechanical axis and b) the amount of force developed by ankle extensor during slow walking In all cases (theoretical and experimental) the results reveal a close correlation between required force muscle length and muscle stiffness The time lag between a change in motor unit discharge and tension development was also calculated to reveal that in many situations (notably gallop) only muscle stiffness can provide a load compensation whereas the delays are too long to allow a reflexly induced load compensation

To understand the kind of commands required of the CNS for the execution of different movements the magnitude of forces involved in each movement must be understood (cf Bernstein 1967 and Gray 1944) Also important is information concerning muscle and motor unit behaviour in the muscle length ranges used in the movements and appreciation of the time lags between a change in the frequency of motor unit discharge and the resultant change in muscle tension

In this report the force required from the ankle extensors to maintain various body positions under postural conditions was derived theoretically It was also possible to derive the force required of these muscles during locomotion The results obtained are then compared to experimental results involving the length ranges at which a muscle exhibits a larger stiffness with different rates of motor unit activity

Particular attention was directed to the motor unit frequencies and muscle length ranges as used in locomotion (Severin, Shik and Orlovski 1967, Engberg and Lundberg 1969) in contrast to those rates and lengths involved in the stretch reflex of the decerebrate cats.

In a previous study (Grillner and Udo 1971 a, b, cf. Granit 1958, Brown 1960) it was shown that in the "rectilinear" range of the stretch reflex of a decerebrate cat the increase in tension with increasing length is mainly an expression of the properties of the contracting muscle. It is an advantage for the control system to have this stiffness built into the active muscle (cf. Rack 1970) since load compensation can then be achieved for those disturbances which must be conciled in a time too short for operation of the admittedly rapid stretch reflex loop (cf., however, Hammond, Merton and Sutton 1956). The present results extend the earlier findings and emphasize the importance of active muscle stiffness as a load compensating mechanism during the stance phase of stepping. The increased force developed by ankle extensors in the yield phase of the step cycle can be ascribed largely to the muscle stiffness itself rather than operation of a set of proprioceptive reflexes. This finding was also supported by the data on the lag between a change of motor unit frequency and a change in muscle tension. This information gives insight into the timing of a potential reflex contribution to the control of stepping. It was concluded that in fast movements such as gallop a reflex effect initiated in one phase of the step cycle cannot affect the tension developed in the same phase but rather is needed for subsequent phases of the movement.

Methods

Eight 2.5—3.5 kg cats were operated under ether and maintained thereafter on pentobarbital sodium given i.v. The left hindlimb was denervated except for the muscle under study (soleus and lateral gastrocnemius). For the soleus experiments (n = 6) lateral gastrocnemius (LG) and plantaris were removed and the tendon of medial gastrocnemius (MG) cut away from the remaining soleus tendon. Soleus was then dissected as free as possible but leaving the origin intact. For LG experiments the soleus tendon was cut away from the Achilles tendon. Plantaris and MG were left in situ so as not to change the mechanical conditions for LG. Then those muscles were carefully dissected free of surrounded connective tissue, except for the measurements shown in Fig. 4. Muscle length was measured in situ from a point proximal to the origin of soleus to a point on the achilles tendon proximal to the level of calcaneus with a pair of compasses; the points for measurements were indicated by a tiny suture sewn into the tendon. The muscle length was measured at maximal flexion (approx. 30°) in the ankle when the foot was forced into maximal ventroflexion and at 90° in the ankle and at maximal extension. In this way a very accurate value for the maximal physiological extension was obtained. After these measurements the calcaneus was cut to leave a small piece of bone connected to the inserting tendon. The tendon of the muscle under study was connected to a strain gauge mounted on a puller which performed accurate changes of muscle length (within 2 μ) at a constant velocity of 0.8 mm/s.

A lumbar (L3—L7) laminectomy was performed and dorsal roots L6 to S1 separated from the spinal cord. Ventral roots L7 to S1 were also cut as close to the cord as possible. The tibia was fixed rigidly and the cat was mounted in a sturdy frame and paraffin oil pools were arranged for the lumbar cord and sural muscles. Pool temperatures were maintained between 35.5° and 38° C and rectal temperature was regulated at 37° C.

Those of the L7 and S1 ventral rootlets supplying α axons to the muscle under study were subdivided into 5 filaments such that stimulation of each produced approximately the same muscle tension (see Rack and Westbury 1969). The 5 filaments were mounted on individual bipolar stimulating electrodes. An additional electrode was placed on the muscle nerve such

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In this report the force required from the ankle extensors to maintain various body positions under [illegible] conditions was derived theoretically. It was also possible to derive the [illegible] required of these muscles during locomotion. The results obtained are then compared to experimental results involving the length ranges at which a muscle exhibits [illegible] stiffness with different rates of motor unit activity.

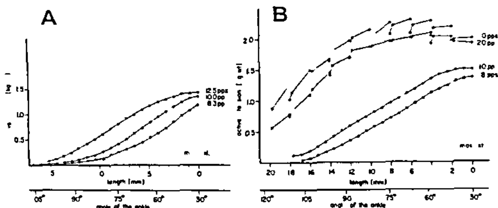


Fig 1 *Active tension developed by soleus at slow extension when stimulated at various frequencies* The left graph shows the active tension (i e passive tension has been subtracted from the total measured tension) versus muscle length expressed in negative values from the max ext (*cf* Methods) and in joint angles The tension was measured by slow extension at 0 8 mm/s while the muscle was being continuously activated b distributed electrical stimulation (*cf* Methods) of the ventral roots at different mean rates The right graph shows the corresponding finding from another soleus muscle To avoid fatigue (40—70 p/s) this muscle was stimulated only for 2 s before it was stretched 2 mm at the same slow velocity The initial value and after 0 25 s and at the end of the stretch are given for each period of stretch These values are connected with fine lines Note the different scales on the ordinates of right and left side graphs The cat in the left graph was small 2 7 kg and thus might the relation between joint angles and length be somewhat smaller since the transscription from length to joint angle is from Rack and Westbury (1969) and not from the actual preparation The slight decrease of the slope for 8 p/s in the right graph only occurred in this preparation

The muscle length obtained in negative values (mm) from the maximal physiological extension can be transferred into joint angles by reference to Rack and Westbury (1969) The strech reflex in the intercollicularly decerebrated cat with no spontaneous α motoneurone activity is usually initiated at a distance of between 10—15 mm from full extension (as indicated in the graphs of Granit 1958 Matthews 1959 a b) which corresponds to ankle joint angles between 90°—30° The length range used during locomotion is quite different Ankle angles ranged from 130° to 80° when the extensors were active (Manter 1938 Engberg and Lundberg 1969) During quiet standing ankle angle approximates 120° (Denny Brown 1928)

From these graphs it is evident that at low frequencies of motor unit activity the active tension in the locomotion range will be small In the length range used during the stretch reflex however considerable active tension is developed as the length is progressively increased up to maximal extension This confirms the results of Grillner and Udo (1971 a b) who showed that the steep portion of the stretch reflex is essentially due to the stiffness of the contracting muscle fibres (motor unit mean frequency at full extension 7 8 ± 1 0 S D) During locomotion extensor motoneurones also of the slow type become active at a much higher rate (15—55 p/s) (Severin Shik and Orlovskii 1967) At such higher frequencies of activity the steep portion of the length active tension curves of Fig 1 A B are shifted o

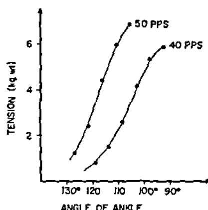


Fig 2 *Active tension developed by the lateral gastrocnemius during slow extension* Data represented as in Fig 1 The angle of the knee joint was 110

coincides with the locomotion range of muscle length Hence a decrease in ankle angle during locomotion will result in an increase of muscle length which automatically gives rise to an increased force to counteract the increased load

Presumably soleus takes part in locomotion in the cat (Engberg and Lundberg 1969) although it is most likely that the gastrocnemii are of greater importance for the force development during locomotion To test if the stiffness of these muscles is also large in the locomotion length region an experiment corresponding to that shown in Fig 1 B was performed on lateral gastrocnemius (Fig 2) Since fatigue is a prominent phenomenon in the muscle (*cf* Burke *et al* 1971) long interval (minimally 4 min) were used between successive periods of stimulation Fig 2 shows a marked increase in active muscle stiffness (1200 g wt/mm) in the locomotion region at stimulating frequencies of 50 p/s and 40 p/s

b) *The required muscle force in the ankle extensors under different mechanical conditions*

Muscle force at different muscle lengths is significant only if it is considered in relation to the resulting torque in the ankle joint This torque varies in relation to the joint angle and to the axis along which the load is applied

Fig 3 A shows a model of the cat s hindlimb in which the limb itself has a small mass and the friction in the joints has been considered neglible In this hypothetical case the cat s hindlimb carries a load (F) through a mechanical axis vertical to the ground crossing the hip pivot and the footpads and estimated to end in line with the end of the metatarsal bones With this model it is possible to calculate the force required from the ankle extensors when the angle between the vertical axis and the metatarsal bones (*i e* Iα in Fig 3 A) and the angle of the ankle joint *i e* IA in Fig 3 A) is changed maintaining the vertical axis through the hip joint and at the end of the metatarsal bones The different lever arms and distances from the ankle joint are indicated in small letters in Fig 3 A

The torque (M) acting at the ankle (*cf* Gray 1944) is a function of the reaction

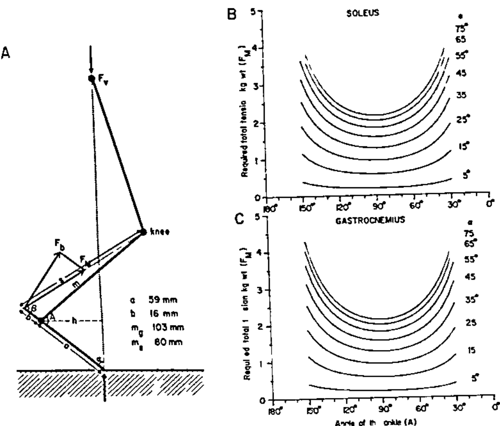


Fig 3 *Required muscle force for some different mechanical conditions* In A a cat s leg is schematically represented with a mechanical axis through the hip pivot to the end of the metatarsal bones A load (F_V) is applied Symbols are given in the text In B and C are the forces required of soleus and gastrocnemius to maintain a particular position of the limb when a load (F_V) of 0 6 kg is applied along the vertical axis Ordinate required muscle force (F_M in eq 7) abscissa angle of the ankle (∠A) This relation has been calculated for different values of the angle between the vertical axis and the metatarsal bones (∠α)

of ground in number equal to the vertical force (F) and the shortest distance between the vertical axis and the ankle pivot (h) which is dependent on the angle α

$$M = F \quad h = F \quad a \sin \alpha \tag{1}$$

For maintaining stable conditions an opposing torque of the same magnitude as M must be developed by the ankle extensors which insert on the tip of calcaneus with a lever arm (b) to the ankle pivot The force (F_b) acting at right angle to this lever arm (b) produces this torque and thus is

$$F \quad a \sin \alpha = F_b \quad b \tag{2}$$

$$F_b = F \quad \frac{a}{b} \sin \alpha$$

The force that the ankle extensors must develop under these conditions (F_M) is dependent on the angle of the ankle joint which changes the muscle length (s) and the angle between F_M and the vector F_b From simple trigonometric functions including the distance from the ankle pivot to the muscle origin (m) the actual muscle length (s) at different joint angles can be obtained

$$s^2 = m^2+b^2-2\,mb\,\cos(180\text{-}A) \tag{3}$$

$$s = \sqrt{m^2+b^2-2\,mb\,\cos(180\text{-}A)}$$

and since

$$\frac{\sin\beta}{m} = \frac{\sin(180\text{-}A)}{s} \tag{4}$$

it follows by substitution of (3) that

$$\sin\beta = \frac{m\,\sin(180\text{-}A)}{\sqrt{m^2+b^2-2\,mb\cos(180\,A)}} \tag{5}$$

and since $\sin\beta$ also equals F_b/F_M it follows that

$$\frac{F_b}{F_M} = \frac{m\,\sin(180\,A)}{\sqrt{m^2+b^2-2\,mb\,\cos(180\text{-}A)}} \tag{6}$$

and by substitution of equation (2) it follows that

$$F_M = F\;\frac{a}{b}\;\sin\alpha\;\frac{\sqrt{m^2+b^2-2\,mb\,\cos(180\text{-}A)}}{m\,\sin(180\,A)} \tag{7}$$

With the help of this equation (7) the force (F_M) required from the triceps surae for maintaining a given position can be calculated at any given load (F_v) applied along the vertical axis

In Fig 3 B F_v is chosen arbitrarily as 600 g wt which would correspond to the load on one hindlimb of 3 kg cat if it carries 20 % of the weight on each hindlimb This is a reasonable estimate in quiet standing (cf Brookhart et al 1965 and also Manter 1938) The length of the different lever arms and distances (a b) are taken from Goslow and Stuart (1972) The distance from the ankle pivot to the origin of the muscle (i e m) was calculated to the middle point of the soleus origin (i e m_s) and to the most proximal part of the tibia (m_g) for gastrocnemius It must be realized however that the gastrocnemius inserts onto the distal femur and that simultaneous knee flexion will decrease the length of the gastrocnemii to some degree In Fig 3 B the force that soleus would have to develop in order to exactly counteract a vertical load of 600 g is plotted at different angles between the vertical axis and the metatarsal bones (i e ∠A) The corresponding graph for gastrocnemius is 3 C The necessary torque at the ankle increases with an increased angle α since the distance between the mechanical axis and the pivot increases progressively It is of considerable interest however that in the 110 —70° ankle angle range the required muscle force is at a minimum and relatively constant

It is striking that the range of ankle angles in which the least force is required i e 120°—80° coincides with the range used in locomotion (Engberg and Lundberg 1969) and for quiet standing (120° according to Denny Brown 1928) The required force increases steeply outside this range of ankle angles

The next calculation involves taking the same load of 600 g on the vertical axis (F_v), and considering what would happen if an extra load of 100 g (∆F) is added to the previously stable position wherein ankle angle is 115° (∠A) and ∠a is 15 This position requires a force of 750 g in the ankle extensors prior to application of the extra load (Fig 3 C) If this tension (750 g) was provided only by the lateral

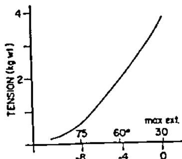


Fig 4 *Passive tension of the triceps surae at different muscle length* The passive tension was measured during slow extension after cutting the muscle nerve The cal caneus was cut and the tendon with its bony insertion connected to the strain gauge No further preparation of the triceps surae was performed and all normally oc curring connective tissue was left in situ the knee angle was 80

gastrocnemius muscle the muscle itself would give a stiffness of 110 g/degree in the ankle joint independent of proprioceptive control (Fig 2) If the angle a did not change the extra load (100 g) would result in a decrease of the angle A with approximately one degree since the stiffness is 110 g/degree which means that gastrocnemius at this angle will produce more than 850 g (Fig 3 B—C) An increase of a is also expected however and this would result in a somewhat increased re quired force If a increased 1° an extra 45 g would be required This can be achieved by lengthening the muscle *i e* decreasing the angle A another half degree to make use of active muscle stiffness Under these conditions the extra load of 100 g (ΔF) on the limb would be counteracted by a decrease in ankle angle of 1 5° including a simultaneous increase of Δa with 1° (*i e* the new values of ΔA = 113 5 and Δa = 16°) This example serves to illustrate that the inherent muscle stiffness can act quite effectively as a length stabilizing system

The situation outlined in Fig 3 corresponds to postural conditions when it can be presumed that slow rather than fast muscle fibres of the gastrocnemii are active and there is greater relative activity in soleus than gastrocnemius While conceding that the natural condition is more complex than the model the theoretical treatment nonetheless serves to bring out the effectiveness of active muscle stiffness as a load compensating mechanism

Only the total tension produced is important for the torque development at the joints It does not matter to what an extent this tension is produced by active con traction in contrast to passive tension Passive tension is mainly due to the connec tive tissue both within and around the muscle A substantial dissection was performed in most experiments to free the muscle from its surrounding attachments (*e g* Rack and Westbury 1969) A minimal dissection is required to evaluate the significance of the passive tension under physiological conditions In one experiment the cal caneus was cut and the triceps surae tendon with its bony insertion was connected to the puller without any further dissection (knee angle was 80°) Passive tension was plotted against ankle angles of 90° to 30° (Fig 4) This simple procedure reveals that passive tension can be sufficiently large to add significantly to the active tension In the 90°—30° length range the force required to counteract a given load increases steeply with a small change of ankle angle and the angle with the mechani

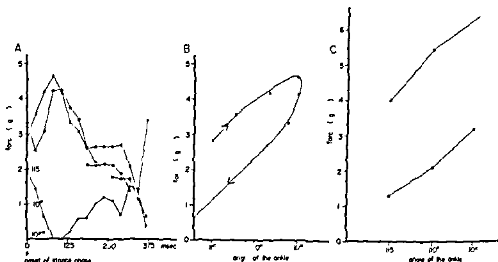


Fig 5 *Relation between muscle length and force in slow walking* The left graph shows the tension developed in the triceps surae during one step cycle (upper curves) The lower curve shows the joint angles in the ankle (note separate ordinate) The data has been calculated from Manter (1938) as discussed in the text The short curve (open circles) between 00—300 m shows the force values obtained from Manter's data (crosses) if compensation is not made for foot ventroflexion In between each point is 1/38 s The middle graph shows the same data (ordinate force abscissa joint angle in the ankle) The right graph shows for comparison the tension developed by the T C when stimulated at 50 (upper) and 10 p/s as obtained in Fig 2

cal axis (Fig 3 B and C *i e* *la* increases and lA decreases) It is valuable to have such an additional load compensating mechanism available at a length range outside the range used in locomotion or posture Passive tension in the triceps surae depends also on the angle in the knee joint With full extension in the knee joint it is impossible to reach a full flexion in the ankle but with increasing degree of flexion in the knee joint it is progressively more easy to reach this position

c) *Locomotion*

The forces developed by the ankle extensors during locomotion can be calculated with the aid of data obtained by Manter (1938) His study includes the vertical and horizontal forces developed by the limbs during slow walking Joint angles (lA) at different stages of the locomotion were also measured For the same stepcycle he presented the forces developed by the hindlimb and thus the direction and size of the mechanical axis (F) as well as its angle in relation to the metatarsal bones (*i e* *la*) If the mass of the foot (1 % of the body weight) and consequently the inertial forces are neglected his data can be entered in eq (7) (*i e* F lA and *la* for subsequent phases of the step) This approximation can be justified for the stance phase of slow walking when acceleratory effects are small (*cf* Manter 1938)

In the slow walk stepcycle of Manter (1938) ankle angles varied between 122°—105° and the angle *a* (*i e* between metatarsal bones and the mechanical axis) between 21°—48 5° The resultant forces varied between 420—1485 g Fig 5 A shows

ankle angles (lower curve) and the force developed by the ankle extensors (filled circles) in subsequent phases of the step cycle (intervals of 1/38 s) After the initial large joint angle at the onset of the stance phase (*i e* when the foot is placed on the ground), the characteristic increase in muscle length takes place (*i e* the yield phase) which again is followed by a muscle shortening Maximal force (4 3 kg) is developed at the peak of the yield and the profiles of the force development and length change in the yield appear as mirror images of one another

For the same stepcycle Manter (1938) approximated the torque in the ankle from free body diagrams taking inertial forces also into account His torque values are transformed into required muscle force (*cf* eq 7) and represented as crosses in Fig 5 A The force values obtained in this way do not differ markedly from those presented above except for some difference in the later part of the stance phase (200—300 ms) This discrepancy results from differences in the point at which the foot is judged to touch the ground

Fig 5 A compares the profile of muscle length (joint angle) and force development for the stance phase of stepping The increase of muscle length (10°) corresponds to approximately 2 5 mm and from Fig 1 B and 2 it is evident that this increase of length will in itself lead to an increased force in the muscle length and motor unit frequency ranges used in locomotion The data in Fig 5 A are plotted as force versus muscle length (*i e* angle in the ankle) in Fig 5 B to emphasize the close correlation between these parameters The force values were obtained in time as indicated by the direction of the line Apparently a hysteretic phenomenon occurs with larger tension during lengthening than during the subsequent shortening A similar hysteresis is exhibited by a muscle during constant motor unit activity if it receives similar sinusoidal length pertubations at similar frequences (velocities of stretch) (Rack 1966) If muscle stiffness is a significant factor for regulating muscle length at different loads one would expect this correlation between length and force provided that the stiffness of the muscle fibres is similar to the slope of Fig 5 B The nature of motor unit activation in stepping is unknown but to illustrate the order of magnitude of muscle stiffness the force produced by the lateral gastrocnemius muscle in this length range (Fig 2) has been plotted in Fig 5 C

If a substantial number of lateral gastrocnemius motor units had been activated at constant frequency some time prior to the stance phase and had then been exposed to the different forces encountered in locomotion (Fig 5 A B) it would be expected that length changes similar to the ones encountered in locomotion would really occur Since it is well established that the motor units of hindlimb extensors do become active well before the foot is placed on the ground and that no marked increase in the integrated EMG occurs (Engberg and Lundberg 1969) it follows that a major portion of load compensation after the foot reaches the ground can be attributed to the stiffness of active muscle rather than a reflexly induced effect (*cf* further below) Increase in force can occur also from motor units recruited later in the locomotor cycle or units still approach[illegible] tension these factors essentially being preprogrammed

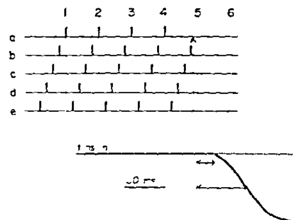


Fig 6

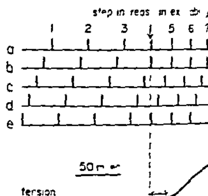


Fig 7

Fig 6 *Effects of sudden stoppage of motoneurone firing on muscle tension* Horizontal Lines indicate the different channels of distributed stimulation and can be looked upon as 5 asynchronously active populations of a motoneurones whose "typical" spikes are represented by the vertical bars Muscle tension is represented below At the arrow all firing stops The interrupted line indicates where the next spike (a 5) was expected to occur to maintain the steady tension. From this point it takes 20 ms before the tension decreases (short double arrows) Duration of half reflaxation time indicated by the long arrow

Fig 7 *Effects of a sudden step increase in excitatory current to tonically active motoneurones on muscle tension* Data represented as in Fig 6 The interrupted line indicates the instant when a step increase in excitatory current occurs This increase allows the motoneurone to discharge with a shorter interspike interval (as performed experimentally the mean rate was changed The excitability in e is such that a spike occurs just when the current is applied (i e the occurring interspike interval (e 4—5) is of the duration that will occur from then on) In channel b spike 4 was apt to occur when the excitability was increased and only the next interval (b 4—5) will be of the new duration

The significance of muscle properties in the reflex time

a) *Inhibitory effects*

In a tonically contracting muscle a sudden stoppage of all motoneurone discharge leads to a gradual decrease of tension How fast this decrease is accomplished will depend on the contractile properties of the motor units (slow versus fast motor units) and the length tension conditions of the muscle at the moment of stoppage

Let us first consider a muscle with a synchronous activity as in Fig 6 but simplified to 5 efferent channels firing at the same mean frequency This efferent activity will produce a smooth tension If all firing stops as indicated tension is still developed by some of the discharge set up immediately before the moment of stoppage

To obtain an estimate of this efferent contribution to reflex time (a-efferent stoppage to drop in muscle tension) the delay from the first inhibited spike (a 5) to the initial drop in tension was measured in a few experiments The lateral gastrocnemius muscle was stimulated with distributed stimulation (as in Fig 1 and 7 and outlined in Fig 6) in order to obtain a smooth contraction When the distributed stimulation was stopped the latency from the first stimulation (i e a 5) that would

have taken place if the stimulation had not been stopped to the initial decrease in tension was measured This is an expression for the minimal efferent contribution to the reflex time This efferent delay was found to be around 20 ms (cf Fig 6) It could vary somewhat (18—30 ms) with changes in initial tension and muscle length as well as mean frequency of distributed stimulation This delay to the first detectable change in tension would probably be longer if it was not concerned with the transition from a continuous activity to complete stoppage but rather with a moderate change of mean frequency of stimulation (e g 40—35 p/s)

The delay from the first missing spike (a 5) to the stabilization of tension at a new mean level will be much longer in the example above the half relaxation time was 60 ms (i e the time to a decrease to 50 % of the initial tension)

In the gallops recorded by Engberg and Lundberg (1969) the stance phase had a total duration of 100 ms and the EMG activity of gastrocnemius started to decrease slightly after 50 ms Some activity persisted however even until the end of the stance phase This indicates that some tension must remain in the ankle extensors in the beginning of the swing phase which the flexors must overcome It is noteworthy that the ankle flexors start their activity (at least in walk and trot) some 30—40 ms prior to the onset of the swing phase (Engberg 1964) Judged from the EMG records of Engberg (1964) and Engberg and Lundberg (1969) there must be tension both in flexors and extensors in the transition both from swing to stance phase and also vice versa

b) *Excitatory effects*

To calculate the delay from an increase in α efferent activity to an increase in muscle tension 2 main possibilities should be recognized First a passive muscle becoming active This involves measurement of the lag between the first spike and the increase in tension and second an active muscle becoming more active This involves measurement of the lag between the first spike discharged at a new and shorter interval and the increase in muscle tension

Fig 7 illustrates the second possibility and shows schematically the delay from the first spike that is discharged at a new shorter interspike interval (e 4) to the onset of an increase in tension This shorter interspike interval is due to a hypothetical step increase in excitatory current (occurring at the arrow in Fig 7) causing a new stable and shorter interval This lag varied between 19—25 ms for LG in 2 expts with distributed stimulation tested with mean frequencies between 8 and 50 p/s

Another factor to be considered is that the activity in the other channels (a—d) is delayed in relation to the activity in channel e Hence the first spike occurring after the new interval will appear later in these channels and only after a period of the same duration as the new stable interval will all motor units have discharged their first interval with this new frequency This is evident if one considers that the last spike to discharge at the new interval will be the one that just before the step increase in excitatory current discharged a spike

Thus only at a delay of 20 ms + the duration of the new interval will all motor units have contributed some tension related to the new interspike interval In muscle activity involving low motor unit frequencies such as the stretch reflex this factor will be very important If the motor units are continuously active and their mean interval changed from 120 to 100 ms it will take 100 ms before all motor units have discharged their first spike with the new interval and 120 ms before the last motor unit has started to contribute its share to this increase in tension at the new frequency During locomotion the interspike interval is much shorter and thus additional lag correspondingly less pronounced

Discussion

General considerations with particular references to locomotion

This paper gives insight into the force requirements for ankle extensors under defined mechanical conditions The relationship between required force and muscle properties has been explored together with the relations between muscle force motor unit activity and muscle length At low rates of motor unit discharge the muscle develops considerable stiffness and tension but only at a length range beyond that used for posture and locomotion At higher rates of motor unit activity however the greatest stiffness is developed within a length range actually used in locomotion. Since these higher motor unit rates are also within the range of those encountered for locomotion (Severin Shik and Orlovski 1967) it has been postulated that the stiffness of active muscle is a load compensating mechanism of its own right with a particularly important role during the stance phase of locomotion

In the present experiments the rate at which the muscle was lengthened was 0 8 mm/s and very much lower than that occurring in locomotion In the yield phase of walking (that pertains to the data treatment in Fig 5) the ankle extensors are stretched at approximately 30 mm/s and this rate increases to over 450 mm/s during fast galloping (Goslow and Stuart 1972) At such high stretch rates muscle viscosity should account for an even larger increase in stiffness than presented in this report (*cf* Rack 1966 1970 Joyce Rack and Westbury 1969) Experiments are in progress to measure these factors in the physiological range of muscle stretch and with application of force inputs of similar magnitude and time course to those occurring in natural movements Also of relevance to future work (particularly in relation to the stance phase of stepping) is the recent finding that the force developed during shortening contraction is greater if it is preceded by a short period of active lengthening (*i e* contraction and lengthening) or to lesser degree isometric contraction rather than a short phase of inactivity as in a passive lengthening (Cavagna Dusman and Margaria 1968 *cf* Cavagna 1970) This finding implies that extensors operating on all limbs require less energy to execute the third extension phase of stepping since the shortening contractions are preceded by active lengthening in the second extension (yield) phase It should be cautioned however, that Cavagna's interesting experiments were performed on directly stimulated amphibian muscles in

which case the mode of motor unit activation is unnatural and on man in which case motor unit activation is quite physiological but at unknown rates to the reader In cat soleus the tension developed during active lengthening is lower than the isometric value at corresponding lengths if the rates of motor unit activation are low (i e $<$15 p/s according to Joyce Rack and Westbury 1969) At higher frequencies of motor unit activation, however the active lengthening values tend to exceed isometric tension Thus this active stretch shorten mechanism might indeed operate during locomotion but not during less demanding motor activity

Muscle properties and the stretch reflex

Grillner and Udo (1971 a) showed that the stiffness of a contracting muscle can account for the increase in tension in the steep linear part of the stretch reflex up to maximum physiological extension This finding was confirmed in the present study with fatigue factors controlled in addition It was strikingly demonstrated that a small increase in motor unit frequency resulted in a large increase in tension at the same muscle length and that the steep part of the active tension length curve was shifted towards a shorter muscle length The relevant curves are shown in Fig 1 and they bear a marked similarity to tho e presented by Matthews (1959 b) for the tonic stretch reflex under various conditions He howed that various excitatory inputs (*e g* repetitive stimulation of nerves to an ipsilateral synergist or to a contra lateral muscle) results in the stretch reflex becoming manifest at a shorter muscle length but with the tension/length slope almost remaining the same Similar excitatory inputs were shown by Grillner and Udo (1971 a) to increase the motor unit frequency by a few imp/s (8 imp/s to 9—12 imp/s) In the p esent study it is not then surprising that one of this range of motor unit frequencies should result in the generation of active tension length curves similar to those developed by Matthews

Grillner and Udo (1971 a) also showed that during slow stretch of the muscle at a constant rate (0 8 mm/s) mo or units recruited to the stretch reflex quickly attain a constant firing rate which is maintained up to maximum extension of the muscle On the termination of stretch motor unit frequency diminishes by 1 to 2 imp/s (*e g* from 9 to 7 8 imp/s)

These findings imply that during that phase of muscle extension when all the motor units have been recruited the excitatory and inhibitory stretch evoked reflex effects on the motoneurones are of equal size since a change in the frequency of motoneurone discharge does not occur A aturation effect is not indicated since discharge rate can be increased by concomitant stimulation of peripheral nerves (Grillner and Udo 1971 a and unpubl) At the moment of motor unit recruitment to the reflex however a net stretch-evoked increase in excitatory current must occur When additional excitatory inputs as described above (*i e* Matthews 1959 b Grillner and Udo 1971 a) are added to the stretch reflex the excitability of all the α motoneurones is increased and in some motoneurones a constant discharge will occur also at short mu cle lengths If to this new level of increased motoneuronal excitability the same stretch evoked net excitatory current is added algebraically →

the motor units will fire at constant but higher mean rates up to maximum muscle extension (*cf* Granit 1966) and silent motor units will be recruited at a comparatively shorter muscle length If the additional excitatory input is supplied by stimulation of the ipsilateral nerve to medial gastrocnemius (*i e* while stretching soleus) at group I strength it can be assumed that mainly the α motoneurones are affected whereas stimulation of contralateral nerves would also change the level of γ motoneurone activity (Grillner 1969) On the basis of these various findings it can be proposed that the slope of the active tension length curve for the stretch reflex in its steep rectilinear part is an expression of the number of activated muscle fibres whereas the threshold for this steep portion is an expression of the mean frequency of motor unit discharge

A detailed discussion of the different muscle afferents contributing to the stretch reflex of the decerebrate cat is beyond the scope of this report It bears particular emphasis however that all the experimental findings are consistent with a single excitatory system (*i e* the primary endings of muscle spindles) (Grillner and Udo 1971 a b and unpubl)

Reflex loop time in relation to the step cycle

All available evidence suggests that a central program controls the timing of muscle activation in vertebrate locomotion (*cf* Graham Brown 1911 1914 Gray 1950 von Holst 1935 *cf* Szekely *et al* 1969 Lundberg 1969) Proprioceptive input during stepping can be assumed to modulate the central program in many ways One function that is often stressed is autoregulation of muscle force (*i e* homonymous reflex inputs) *in the same phase of the step in which the input is initiated This possibility* will now be scrutinized

When a sural motor unit is continuously active a time lag of 20 ms or more occurs between a change in motoneurone firing rate and the first detectable change in tension in either direction (Fig 6+7) To this time lag the delay between receptor activation and change in spike activity of the motoneurone must also be added The fastest total reflex time for the ankle extensors must be close to 30 ms and this time would be considerably lengthened for the changes in all the motor units affected to take place and a new steady tension level to be attained

These time lags impose a severe restriction during fast movements such as a top speed gallop The second extension phase (*i e* from the foot being placed on the ground until the maximal yield is reached) is then only 31 ms in duration (Goslow and Stuart 1972) It follows that a reflex effect evoked at the beginning of the second extension phase cannot become evident until the third extension phase begins The maximal force developed however occurs at the peak of the yield (Fig 5) For fast movements it can only be concluded that proprioceptive input affects the subsequent phase to that in which it is developed In slow movements such as slow walking (yield time over 100 ms Engberg and Lundberg 1969 Goslow and Stuart 1972) a reflex control can occur Also contributing to increased reflex effects is the firing rates of the motoneurones themselves In locomotion those rates (15—5

Shik Severin and Orlovski 1967) are partially within Kernell s (1965) secondary range in which a given synaptic current has a greater effect on firing rate than in the primary range of discharge Even in moderately fast walking however the duration of the yield is near 80 ms (Goslow and Stuart 1972) All in all the evidence does not promote the importance of autoregulatory load compensating effects in the control of faster types of locomotion Rather it is suggested that a main role of proprioceptive input (at least in the yield phase) is for executing subsequent phases of the same and successive cycles through its effects on the spinal interneurones responsible for the alternating activation of flexors and extensors and for the interlimb coordination as well as on the midbrain and cerebellum (*cf* Bernstein 1967 Roberts 1967)

In the neuromuscular control of locomotion the load compensating mechanism must operate with great precision By building the mechanisms into the muscle itself inappropriate time lags are obviated Proprioceptive control with its unavoidable time lags (impulse initiation conduction and transmission times and electromechanical coupling) can then superimpose further adjustments Viewed in this light it it not surprising that during phylogeny the visco-elastic properties of muscles have evolved to meet the requirements for natural movements Nor is it surprising to find that during fast movements an effective load compensation cannot be achieved by proprioceptive route It is in no way implied of course that proprioceptive information is not indispensable for the neural control of movement (*cf* Bernstein 1967)

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Effects of Hypoxia and Glucocorticoids on the Histochemically Demonstrable Catecholamines of the Newborn Rat Carotid Body

By

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Abstract

HERVONEN A L KANERVA O KORKALA and S PARTANEN *Effects of hypoxia and glucocorticoids on the histochemically demonstrable catecholamines of the newborn rat carotid body* Acta physiol scand 1972 86 109—114

The histochemically demonstrable catecholamines of newborn rat carotid bodies were studied using the formaldehyde induced fluorescence (FIF) method The effects of both heavy anoxia and repeated injections by glucocorticoids were registered The newborn rat carotid body consisted of bright yellow fluorescing cells During the following 2 weeks the intensity of this fluorescence decreased distinctly while fluorescent network of adrenergic nerve fibres developed The fluorescent nerves were in close contact with the chief cells of the carotid body Hydrocortisone or prednisolone treatment prevented the loss of fluorescence normally occurring Anoxia caused a marked decrease in the FIF in the majority of glomus cells in 9—16 day old rats

Oxygen deficiency and the parallel changes in carbon dioxide concentrations and in the pH levels of the blood are generally regarded as activating stimuli for the chemosensory function of mammalian carotid body (Zapata *et al* 1969 Morita Chiocchio and Tramezzani 1969) The chief cells (or glomus cells) of the carotid body are known to contain catecholamines and 5 HT (Palkama 1965 Hamberger Ritzen and Wersall 1965 Zapata *et al* 1969 Chiocchio King and Angelakos 1971) However the functional importance of these monoamines in eliciting the chemosensory response is still a matter of discussion (for ref see Biscoe 1971)

The changes in the formaldehyde induced fluorescence (FIF) of rat carotid body were studied after repeated injections of hydrocortisone or prednisolone into the newborn animals Both the normal material and the glucocorticoid series were subjected to severe anoxia after which the FIF was demonstrated

Material and methods

Litters of Sprague Dawley rats were used Each experimental series was initially divided into a control group and a glucocorticoid test group The steroids used were the following Prednisolone (as sodium tetrahydrophtalate) ultra corten® H (kindly supplied by CIBA) hydrocortisone acetate (kindly supplied by ORGANON) The doses were 10 mg/kg of b and 40 mg/kg of b w respectively

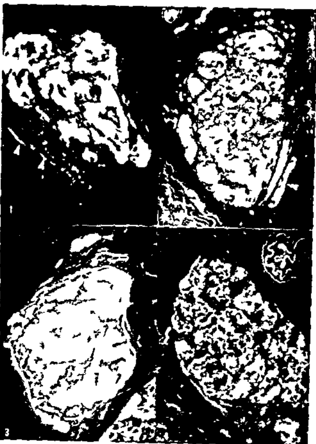


Fig 1 FIF of the carotid body of a 1 day old rat The chief cells exhibit an intensive yellowish fluorescence The organ is surrounded by greenish fluorescent nerve fibres (arrows)

Fig 2 FIF of the carotid body of a 16 day old rat Note the few groups of intensively fluorescent cells still present (black arrows) Thick fluorescent nerve trunk in the right lower corner of the figure (light arrow) 275 x

Fig 3 FIF of the carotid body of a 1 day old rat after severe oxygen deficiency for 2 h The intensity and the colour of the fluorescence are the same as in the control series Slightly more diffusion of the fluorescent amines can be observed 275 x

Fig 4 FIF of the carotid body of a 16 day old rat after severe oxygen deficiency for 2 h A clear decrease in the fluorescence intensity can be observed The brightly fluorescent groups of chief cells visible in the controls have disappeared The adrenergic network of nerves can be detected when the FIF of the chief cells is diminished 275 x

Half the injected and the normal animals were subjected to severe anoxia for 1—2 h The *other half were killed* without any experiments Anoxia conditions were prepared by keeping the animals in tight glas jars filled with carbon dioxide The milieu was oxygenated if needed

The carotid bodies were prepared quickly and were frozen in isopenthane cooled with *liquid nitrogen The freeze* drying procedure was performed following the general rules laid down by Eranko (1967) and Olson and Ungerstedt (1970) The following technical values *were used* drying temperature —37 C drying vacuum 10^{-3} torr drying time 2—3 days exposure to paraformaldehyde vapour 45—60 min at 60—80 C

The specimens were embedded in a mixture of Epon and Araldite resins (Eranko and Eranko 1971) and were cut at 2—3 µm with LKB Pyramitome A Leitz Ortholux microscope equipped with Ploem epi illuminator was used in the fluorescence studies The following filters were used a 3 mm thick BG 38 and a 3 mm thick BG 3 heat absorbing filters a TAL 408 interference filter (all filters by Schott & Gen Mainz) and a Leitz ultraviolet filter K 470 An Osram mercury lamp HBO 200 was used as the ultraviolet beamsource

Results

1 Normal carotid body

1 day old rat The carotid bodies were easily recognizable between the bases of internal and external carotid arteries The chief (or glomus) cells exhibited intensive green to yellow fluorescence which faded away rather slowly A few cells with extremely intensively fluorescent cytoplasm were found among the chief cells The

small groups of fluorescent cells were separated by capillaries and connective tissue elements Greenish fluorescing nerve fibres were seen between the cell groups and also outside the organ in larger bundles (Fig 1)

6 day old rat—The fluorescence intensity of the chief cells had decreased distinctly since the 1-day stage The colour of the FIF had not changed The most intensively fluorescent cells of the organ were still present The total area of the organ remained about the same The number of fluorescent nerve fibres increased clearly during the first week of postnatal life

9 day old rat—The only change on the previous stage concerned the FIF of the chief cells a further decrease of intensity was evident The relative amounts of nerve fibres remained unchanged

16 day old rat—With the further decrease in intensity of FIF its colour also turned closer to green Among the weak green fluorescent chief cells some cell exhibited the intensive yellowish FIF Mast cells were frequently found at this age but the quality of their FIF was not differentiable from the other yellowish fluorescent cells Nerve fibres surrounded the groups of chief cells forming a tight network within the organ (Fig 2)

Effect of anoxia on the normal carotid body

No difference was observed in the carotid of 1 and 6 day old rats when the normal material was compared with the anoxia series (Fig 3) On the other hand in the 9-day and the 16-day old rats the effect of anoxia was detected the green fluorescence of the chief cells decreased greatly, leaving about a half of them totally non fluorescent All the bright yellow fluorescent cells observed in the normal material disappeared (Fig 4) Anoxia had no effect on the nervous network or the mast cells of the organ

2 The effect of corticosteroids

The effect of prednisolone and hydrocortisone was the same Neither qualitative nor quantitative differences were observed when the experimental series were compared However the rats tolerated prednisolone better Occasional deaths of the injected animals were encountered in the hydrocortisone series

6 day old rat—The decrease in FIF which was noticed in the control group was prevented and the FIF corresponded to the FIF of the 1-day old rat

9 day old ra —The intensity of the FIF of the chief cells clearly exceeded that of the control specimens The shift in FIF colour towards green was also prevented by the hormones The FIF of the nerve fibres was not changed

16 day old rat—The hormones prevented a decrease in the fluorescence intensity of the chief cells (Fig 5)

Effect of anoxia on the carotid bodies of the glucocorticoid injected rats

Oxygen deficiency did not produce any detectable changes in the fluorescence intensity oof the chief cells (Fig 6)

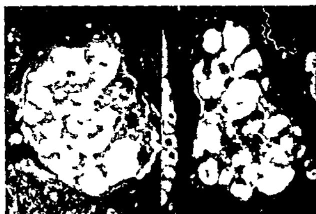


Fig 5 FIF of the carotid body of a 16 day old rat after treatment with prednisolone The fluorescence of the chief cells is as intensive as in the organ of a 1-day old rat 275 ×

Fig 6 FIF of the carotid body of a 16 day old prednisolone treated rat after severe oxygen deficiency No remarkable difference from Fig 5 can be noticed 275 ×

Discussion

1 FIF of the normal carotid body

The greenish or yellow colour of the fluorescence was identical to that found in the adrenal medulla or paraganglia of the same rats The colour of the fluorescence in this case provides no means of differentiating the catecholamines and 5 HT The yellow colour of the fluorescence especially in certain brightly fluorescent cells might indicate the presence of 5 HT However the colour of FIF exhibited by the catecholamines also changes closer to yellow when the amounts of amines were increased (Jonsson 1969) Hamberger *et al* (1965) used the rapid fading rate of the fluorescence as criteria of the presence of 5 HT In the present specimens the fading rate of the FIF was not remarkably different from that of the FIF in paraganglionic or adrenomedullary cells Hence the chemistry of the amine or amines responsible for the FIF of the chief cells remains unsolved

The intensity of the FIF was greatest in the newborn animals and decreased during the following 2 weeks This phenomenon might be related to the similar involution occurring in other parts of the newborn rats paraganglionic tissue Lempinen (1964) suggested that this involution of the paraganglia might be due to the extremely low levels of glucocorticoids in the blood of the postnatal rat The present results of the effects of glucocorticoids of the FIF of carotid bodies support this concept The prenatal function of the carotid body may be similar to that of other paraganglia but after birth the organ presumably assumes a solely chemosensory function loosing any endocrine nature The adrenergic innervation of the organ also seems to develop mainly after birth supporting the idea of change in the basic function of the organ during the perinatal period It is suggested that the rat fetal carotid body might differentiate into an organ storing catecholamines because of the effect of humoral inductive agents which also guide the differentiation of the paraganglia (Hervonen 1971) After birth the diminished influence of the humoral agents probably glucocorticoids led to the decrease in the amine content of the chief cells

The adrenergic innervation of the carotid body originates in the superior cervical ganglion The rapid development of a rather tight network of nerve fibres around

TABLE I Catecholamine fluorescence in the glomus cells of newborn rat carotid body

AGE (days)	1	6	9	16
Normal	xxxx	xxx	xx	x
Normal hypoxia	xxxx	xxx	x	x
Glucocorticoids	xxxx	xxxx	xxx	xxxx
Hypoxia	xxxx	xxxx	xxxx	xxxx

The fluorescence intensity is labelled as follows negative weak x moderate xx strong xxx very strong xxxx

the small groups of chief cells might indicate the maturation of the chemoreceptor functions of the organ Bright varicosities were found in the nerves on the chief cells The role of the sympathetic postganglionic innervation of the carotid body is obscure (Biscoe 1971) In the few papers dealing with the FIF of carotid bodies no valuable comments on the adrenergic innervation of the organ were found The present fluorescence microscopic technique however clearly demonstrates a tight network of fluorescent fibres Judging from the present findings it seems reasonable prenata function of the carotid body may be similar to that of other paraganglia to assume that both the adrenergic innervation might be functional in the postnatal ra ca otid body The real nature of the synapses should be solved using electron microscopic techniques

2 Effect of glucocorticoids

Both hydrocortisone and prednisolone prevented the decrease in fluorescence intensity normally occurring during the first 2 postnatal weeks The effect was mainly similar to that of glucocorticoid on the catecholamine storing cells in newborn rat paraganglia and sympathetic ganglia (Lempinen 1964 Eranko and Eranko 1972 Kanerva *et al* 1972) (Table I) The effect seemed to be limited only to the maintenance of the fluorescence intensity level in the carotid body In sympathetic ganglia and paraganglia elsewhere the effect of the hormones was more marked causing a distinct increase in the total quantity of catecholamine storing elements In the present study the size of the carotid bodies was not affected by the hormone treat m nt

3 Effect of anoxia

The effects of anoxia were detected in the carotid bodies of 9 and 16 day old rats The clear decrease in the fluorescence intensity of the chief cells indicated a discharge of catecholamines from the organ (Table I) Hoffman and Birrel (1958) and Blumcke *et al* (1967) reported parallel results using electron microscopic techniques No studies concerning changes in the catecholamine fluorescence were available The nature of the present method hould be emphasized even extensive loss of catecholamines from the chief cells might have escaped detection because of the intensity of the FIF Hence the unchanged FIF of the carotid bodies of 1

old rats does not exclude the possibility of catecholamine discharge from the organ Zapata *et al* (1969) came to the conclusion that the catecholamines do not play a significant role in the generation of chemosensory discharges However the clear changes in the intensity of FIF observed in the anoxic carotid bodies are probably due to changes involved in the chemosensory response to the oxygen deficiency The authors consider it reasonable to assume that the catecholamines are involved in the mediation of the chemosensory function of the organ Chen *et al* (1969) found no decrease in the granular content of glomus cells after hypoxia in the hamster carotid body Their finding are in accordance with those of Al Lami and Murray (1968) in the cat carotid body, who found that the electron density of the enclosing cells even increased

These observations point out the possibility, that the chemosensory respon e is not directly mediated by the enclosed glomus and their granular content

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Acta physiol scand 1972 86 115—119

From the Institute of Physiology University of Lund Sweden

Release of Acetylcholine in the Parotid Gland of the Dog during Stimulation of Postganglionic Nerves

By

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Abstract

HOLMBERG J *Release of acetylcholine in the parotid gland of the dog during stimulation of postganglionic nerves* Acta physiol scand 1972 *86* 115—119

It has been found recently that the dog s parotid is supplied with postganglionic secretory fibres which travel not only in the auriculo-temporal nerve but also outside this nerve in the adventitia of the internal maxillary artery In the present experiments acetylcholine was found to appear in the perfusate from the gland when the auriculo-temporal nerve was stimulated When both this nerve and the fibres on the internal maxillary artery were excited the yield of acetylcholine was doubled It is concluded that the auriculo-temporal nerve and the nerves on the internal maxillary artery contain cholinergic nerves in about equal amounts

Postganglionic secretory nerves reach the parotid gland of the dog not only via the auriculo temporal nerve but there is also a substantial amount of secretory fibres travelling on the internal maxillary artery (Holmberg 1971 1972) Both sets of nerves seem to belong to the parasympathetic system for stimulation of the cervical sympathetic trunk does not evoke any secretion from the canine parotid (Heidenhain 1878 Emmelin Ohlin and Thulin 1969) Furthermore the secretory effects of electrical stimulation of these postganglionic nerves are abolished by atropine (Holmberg 1971) These nerves thus seem to be cholinergic This assumption has now been supported by perfusion experiments in which release of acetylcholine was found to occur when both sets of nerves or one of them were stimulated electrically The experiments were considered of interest also for the following reason The parotid gland offers one of the few examples of an organ the perfusion of which would not include synapses between pre and postganglionic parasympathetic neurones If perfusion of the vascular tree of the gland could be arranged it would be possible to study acetylcholine release avoiding the complications created by the presence of a ganglionic synapse

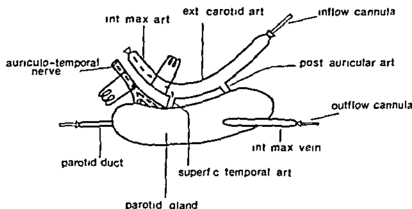


Fig 1 Diagram of the preparation of the parotid gland The course of the secretory nerves to the gland is indicated with dashed lines

Methods

The experiments were made on 6 dogs In 3 of them both parotids were used and in 2 others one submandibular gland was studied for comparison Chloralose urethane (50+500 mg/kg) was given iv after preliminary ether anesthesia and tracheal cannula was inserted The parotid gland was isolated for perfusion in the following way (Fig 1) A midline incision was made on the ventral side of the neck The pterygoid muscles and the biventer muscle were severed Likewise the internal maxillary vein was divided medial to the parotid gland It was then possible to localize the origins from the external carotid artery of the 2 arteries which supply the parotid the superficial temporal and the posterior auricular All connexions between the gland and its surrounding structures were then divided except for these 2 arteries and the internal maxillary vein where it emerges on the lateral surface of the gland The auriculo-temporal nerve was divided at its origin from the mandibular nerve and a long stump outside the parenchyma was saved of the internal maxillary artery which carries postganglionic nerves to the gland in its adventitia A cannula was introduced into the parotid duct The auriculo-temporal nerve and the internal maxillary artery were placed on bipolar electrodes the one on the artery being situated 1—2 mm from the origin of the superficial temporal artery Square wave pulses supramaximal judging from the secretory responses were delivered through these electrodes at a rate of 20/s for periods of 30 s The drops of saliva falling from the cannula were recorded on a smoked drum by means of a manually operated electro-magnetic pen The secretory responses to stimulation of the two nerves separately and simultaneously were first recorded then perfusion was started

Blood plasma was used as perfusion fluid At the beginning of the experiment blood was withdrawn from a femoral artery collected in heparin and centrifuged Eserine sulphate was added to the plasma to a concentration of 10 The plasma was then warmed to 37° C bubbled with oxygen containing 5 % CO_2 and injected from a motor driven syringe into the cannula in the external carotid artery Perfusate from the cannula in the internal maxillary vein was collected in ice-cooled vessels A very low perfusion rate was chosen about 1 ml/min Each sample was collected for 3 min The intervals between the collection periods were not longer than necessary for changing the vessels When nerves were stimulated this was done during the first 2 min of the period Each stimulation sample was preceded by 2 control samples It would have been desirable with more control samples but the limited amounts of plasma available did not allow that The general arrangement was that the two sets of nerves was stimulated together on two occasions and in between there was a sample collected when the auriculo-temporal nerve was stimulated alone

The submandibular glands were similarly perfused from a cannula in the submandibular artery and perfusate collected from a cannula inserted into the largest glandular vein found all other vessels had been tied The chorda lingual nerve was stimulated by a monopolar electrode at a frequency of 20/s with shocks of 2 ms duration and supramaximal intensity

The samples of perfusion fluid collected were kept on ice in a refrigerator Immediately after the perfusion experiment their acetylcholine content was estimated on the blood pressure of

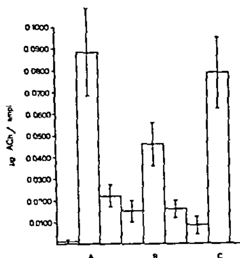


Fig 2 Mean acetylcholine content in perfusion samples collected during 3 min from 9 canine parotids Nerves were stimulated for the first 2 min of period A B and C during B only the auriculotemporal nerve during A and C both this nerve and the nerves on the internal maxillary artery The vertical bars represent ±S E of the mean

small eviscerated cats as described by MacIntosh and Perry (1950) Eserine sulphate 0 05—0 1 mg/kg was given if necessary to increase the sensitivity to acetylcholine Acetylcholine chloride was used as a standard and the figures given refer to the salt Statistical evaluation was carried out with student s t test using paired data for each experiment.

Results

Parotid gland

The secretory responses of the perfused parotid gland were much smaller than those of glands with normal circulation in agreement with general experience The venous effluents were usually free from admixture of blood and the amounts obtained similar to those introduced on the arterial side Judging from the appearance of the gland the whole gland seemed to be reached by the perfusion and indian ink injected into the arterial perfusion cannula at the end of each experiment was evenly distributed in the gland It thus seems possible to produce a perfused preparation of the parotid of the dog

The stimulation samples invariably lowered the blood pressure of the assay cat and since the effect was abolished by atropine (200 µg/kg i v) it was attributed to acetylcholine The first control samples contained acetylcholine only in one case and this amount was very small (0 01 µg) When both nerves were stimulated in period A and C of Fig 2 the acetylcholine yield was 0 09 ± 0 02 and 0 08 ± 0 02 µg/sample respectively (Mean ± S E) About half as much 0 05 ± 0 01 µg/acetylcholine/sample (Mean ± S E) was obtained when only the auriculo temporal nerve was excited during period B In every experiment the amount of acetylcholine in the sample from period B was smaller than the mean amount collected during period A and C This is a significant difference ($p < 0.01$) The acetylcholine released during the stimulation periods was not completely washed out during these periods but appeared in decreasing amounts in the 2 succeeding control periods There was however significantly more in the stimulation periods than in their preceding control period ($p < 0.01$)

Submandibular gland

Stimulation of the chorda tympani released almost twice as much acetylcholine into the submandibular gland as did excitation of both the nerves in the parotid. The acetylcholine content in 4 stimulation samples from the 2 submandibular glands was found to be 0.15 ± 0.03 μg (Mean ± S.E.). Also in one of these glands acetylcholine could be demonstrated in two control samples collected before stimulation. The amounts were small, 0.02 and 0.03 μg respectively, whereas the ensuing stimulation sample contained 0.22 μg ACh.

Discussion

It is wellknown that in most experiments demonstrating the release of acetylcholine on stimulation of parasympathetic nerves the acetylcholine obtained could originate both from pre and postganglionic nerves, owing to the facts that preganglionic fibres were stimulated and fluid for bioassay was collected from tissue containing both synaptic and neuro-effector junctions. There are, however, some preparations in which acetylcholine liberation postganglionically can be studied separately. In the early experiments on the eye made by Engelhart (1931), preganglionic fibres were activated, but acetylcholine was detected in the acqueous humour and could not be derived from preganglionic endings. The urinary bladder of the rat can be isolated without ganglion cells, and acetylcholine was found to appear in the bath fluid surrounding such a preparation on stimulation with field electrodes (Carpenter and Rand 1965, Chesher 1967). As to salivary glands, the submandibular gland contains numerous parasympathetic ganglion cells, and acetylcholine found in a perfusate on chorda stimulation could be of both pre- and postganglionic origin. However, pharmacological blockade of ganglionic transmission greatly reduces the yield of acetylcholine, showing that the transmitter obtained in the absence of the ganglion blocking agent was to a great extent released from the postganglionic neurone (Emmelin and Muren 1950).

In the present experiments it proved possible to perfuse the parotid gland of the dog and to obtain acetylcholine on stimulation of the nerves containing secretory fibres for the gland. There is every reason to belive that the fibres stimulated were exclusively postganglionic: secretion elicited by stimulation of these nerves is not affcted by hexamethonium (Holmberg 1971) and no ganglion cells can be detected histologically within the gland (J. R. Garrett, personal communication).

The amounts of acetylcholine released in the parotid glands were about half of those obtained in the submandibular glands. This is no doubt partly due to the fact that in the submandibular glands acetylcholine had been released from preganglionic endings also. Earlier experiments suggest, however, that only 1/3—1/4 of the total yield of acetylcholine obtained in such glands is of synaptic origin (Emmelin and Muren 1950), and it may be that under comparable conditions less acetylcholine is released postganglionically in a parotid than in a submandibular gland. This would be compatible with the facts that the parotid contains fewer cholinesterase positive

nerves (J R Garrett personal communication) and has a smaller acetylcholine synthesizing capacity than the submandibular gland (Nordenfelt 1964 Ekstrom and Holmberg to be published)

The findings that acetylcholine is released when the auriculotemporal nerve or the nerves on the internal maxillary artery are stimulated do obviously not prove that the secretory fibres are cholinergic other nerves, vasodilator or motor nerves for myoepithelial cells could be responsible for the acetylcholine However they strongly support that view, suggested by the fact that the secretory responses to stimulation of the nerves can be abolished by atropine It is also interesting to note that about the same amounts of acetylcholine can be released on stimulation of the auriculo temporal nerve and the nerves on the artery for the secretory responses obtained in these two cases are about equal (Holmberg 1971)

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Pre- and Postganglionic Secretory Pathways for the Parotid Gland of the Dog

By

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Abstract

HOLMBERG J *Pre and postganglionic secretory pathways for the parotid gland of the dog* Acta physiol scand 1972 *86* 120—125

Recently earlier unknown secretory nerve fibres to the parotid of the dog have been traced outside the auriculo-temporal nerve on the internal maxillary artery and in the facial nerve In the present experiments it was investigated whether they conveyed impulses from the classical preganglionic nerve the tympanic one or from secretory nerves outside this nerve the existence of which has been suggested by previous workers The parotid secretory response to electrical stimulation of the tympanic nerve was found to be successively reduced when the auriculo-temporal nerve the internal maxillary artery and the facial nerve were divided Some additional evidence is presented for the conception that most of the impulses travel in the tympanic nerve and are conveyed to the gland via the auriculo-temporal nerve and the adventitia of the internal maxillary artery Uncharted secretory pathways outside the tympanic nerve were found to contribute very little to the reflexly induced secretory response because after division of the tympanic nerve this response was abolished in 5 glands out of 10 whereas only a slight secretion was obtained in the others

Several observations suggest that postganglionic parasympathetic secretory nerves reach the parotid gland of the dog by some other route than the auriculo temporal nerve (see Holmberg 1971 1972 a b) It was found for instance that reflexly induced parotid secretion was not abolished by section of this nerve but only reduced to about half of the normal (Holmberg 1972) Recently it was observed that the secretory response remaining after auriculo temporal nerve section can be greatly diminished or even abolished by cutting nerve strands on the internal maxillary artery and electrical stimulation of these strands caused a flow of saliva which was not affected by ganglion blocking agents but disappeared after atropine injection (Holmberg 1971) Hence it can be concluded that the nerves on the artery contain numerous postganglionic cholinergic secretory fibres for the parotid gland In addition a small amount of secretory fibres seems to travel in the facial nerve (Ekstrom and Holmberg to be published)

The preganglionic connections of these new secretory pathways have not been explored The tympanic branch of the glossopharyngeal nerve is generally assumed

to contain the preganglionic supply to the otic ganglion (Loeb 1869) but observations made by Andreyev and Podkopayev (1928) indicate that there are other preganglionic pathways with secretory nerves for the parotid gland for these investigators found that the reflexly elicited secretory response of the gland was not abolished by cutting the tympanic nerve but only reduced by 75—80 %

In the present investigation the relationship between the tympanic nerve and the various postganglionic nerves was studied This was done by recording the secretory responses to electrical stimulation of the tympanic nerve before and after section of the different po ganglionic nerves and also by comparing the responses obtained on tympanic nerve stimulation with those produced by stimulation of the combined postganglionic nerves Since the observations made in these experiments suggested that the known postganglionic nerves at least to a great extent receive their secretory impulses from the tympanic nerve and since section of these postganglionic nerves usually abolishes or almost abolishes reflexly induced secretion (Holmberg 1971 Ekstrom and Holmberg 1972) it seemed desirable to try to assess the quantitative importance of the preganglionic fibres outside the tympanic nerve the existence of which can be postulated from the experiments described by Andreyev and Podkopayev (1928) For this purpo e the effect of section of the tympanic nerve on the reflexly induced secretory responses was investigated

Methods

Electrical stimulation of the tympanic ne ve before and after division of postganglionic nerves

6 dogs were used The animals were anesthetized with chloralose urethane (50 + 500 mg/kg) i v after induction with ether Tracheal cannula was inserted The parotid duct was cannulated with polyethylene tubing and was connected to an overflow bottle where saliva displaced water The drops of water falling from the outlet of the bottle were recorded with ordinate writers or electromagnetic pens on a smoked drum Four animals ere cannulated unilaterally and two bilaterally The head of the animal as fixed to the table using specially constructed clamps and the bulla of the middle ear was opened as described by Heidenhain (1878) By means of a simple micromanipulator electrodes were pushed down between the wall of the tympanic cavity and the tympanic nerve They consisted of two straight platinum strings 0 1 mm in diameter insulated except for a length of 1—2 mm on the side in contact with the nerve Square wave shocks of 2 ms duration were applied to the nerve at a rate of 20/s using supramaximal intens ty The duration of the stimulation periods was from 1 min for high secretory rates to 3 or 5 min for lo ones The pause between the periods was at least 5 min The secretory responses to stimulation of the tympanic nerve were recorded before and after acute division of the auriculo-temporal nerve on the medial side of the mandibular joint of the internal maxillary artery at the same level and of the facial nerve ju t outside the stylomastoid foramen In two of the animals the internal carotid artery the agal and glossopharyngeal nerve and the sympathet c trunk were also divided as close to the jugular foramen as possible When many nerves had been severed and the secretion recorded was very scanty the secretory effect of the stimulation of the tympanic nerve had to be enhanced in some cases by the local administration of eserine to the gland Eserine sulphate 3 μg or 30 μg in 0 3 ml saline was then injected into the parotid duct us ng the method of Emmelin Muren and Stromblad (1954) The eser ne often caused secretion from the gland within 1—2 min Then the nerve was not stimulated until the secretion had markedly diminished to be able to detect the possible effect of the stimulation as an a celeration in the salivary flow When no secretion followed the injection stimulat on was started after 5—10 min

On one side in the 2 animals which had been cannulated bilaterally a control experiment was made the tympanic nerve was stimulated before and after section of the nerve distal to the stimulating electrodes In this way the influence of irradiation and centripetal stimulation on the secretory responses could be as essed

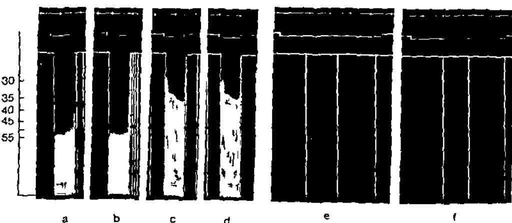


Fig 1 Parotid secretory rate in response to electrical stimulation of the tympanic nerve (70/s) before (a and b) and after division of the auriculo temporal nerve (c and d) first and then the internal maxillary artery (e and f) Tracings from above time in min signal drops of saliva recorded with ordinate writer the calibration of which is shown left upper row in drops/min One ml gave 31 drops

The frequency response curve of the tympanic nerve

11 glands from 8 dogs were used On 2 separate days the maximal reflexly inducable secretory rate was determined in light thiopentone anesthesia as described earlier (Holmberg 1971) After at least one day of rest the animals were prepared and the tympanic nerve was timulated as described above The excitation period however was 1 min in all these periments and the stimulation frequency was varied between 40 and 1 shock/s starting with e higher frequencies and successively going down The stimulation periods were interrupted by periods of rest for 5 min when excitation frequencies higher than 8 shocks/s were used and for 2 min when lower rates were employed The most rapid secretory rates recorded during 30 s were expressed as percent of the highest salivary flow rates obtained during 30 sec of reflex activation

Denervation experiments

10 dogs were used On 2 separate days the maximal reflexly induceable secretory rate was determined in light thiopentone anesthesia The second time the anesthesia was deepened with an additional dose of thiopentone An endotracheal tube was then introduced and the anesthesia maintained with ether The tympanic nerve was unilaterally divided with a knife close to its entrance into the tympanic cavity on the promontory One animal was sham operated with destruction of the mucous membrane of the tympanic cavity but preservation of the tympanic nerve On the 3rd to 6th postoperative day reflexly induced secretory rate was then recorded as described above In the sham operated animal this was also done on the 14th and 21st day

Drugs

Hexamethonium bromide (20 mg/kg) and atropine sulphate (200 μg/kg) were given i v in some of the acute experiments The same dose of atropine sulphate was also administered intracardially during and methacholine 1 or 2 μg/kg before or after reflex activation

Results

The course of the secretory pathways from the tympanic nerve

The well reproducible secretory response obtained when the tympanic nerve was stimulated, was abolished after hexamethonium 20 mg/kg Thus the nerve fibres stimulated were exclusively preganglionic It was also abolished when the tympanic

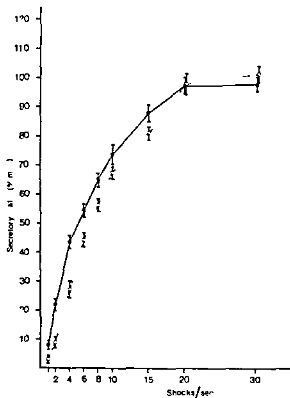


Fig 2 Continuous line Relation between parotid secretory rate and stimulation frequency in the tympanic nerve in 11 parotid glands obtained in the present report Dashed line the corresponding relation for the simultaneous stimulation of the auriculo-temporal nerve and the nerves on the internal maxillary artery in 9 parotids from a previous report (Holmberg 1971) Ordinate secretory rate in % of the maximal rate that could be induced reflexly Abscissa Stimulation frequency The vertical bars represent ± S E of the mean

nerve had been divided distal to the stimulating electrode indicating that it was solely mediated by impulses travelling from the electrode in the distal direction in the tympanic nerve The response was greatly reduced after section of the auriculo-temporal nerve In the typical experiment shown in Fig 1 the initial response was reduced by 40 % and in the others by 71, 67 53 45 and 25 % respectively Hence a large but variable part of the secretory pathway from the tympanic nerve was present in the auriculo-temporal nerve The main remaining part of this pathway evidently had its course on the internal maxillary artery because when this vessel had been divided as well the response to stimulation of the tympanic nerve was further reduced as illustrated in Fig 1 In one experiment out of 6 5 % of the initial response was still obtained in the others below 1 % These two nerves were not the only ones which could be activated from the tympanic nerve because a further reduction in the very small remaining secretory response to stimulation of this nerve was obtained in all 6 expts when the facial nerve had been divided in addition to the two other sets of nerves In 3 of 6 animals the response was totally abolished by this procedure and in 2 others secretion could be provoked only in the presence of eserine In 2 of the animals where a slight secretion was provoked even after division of all 3 nerves mentioned above a further denervation of the gland

attempted Thus the vagal and glossopharyngeal nerves the sympathetic trunk the internal carotid artery were severed close to the jugular foramen The etory responses appeared to be slightly reduced after this maneuvre but they e not completely abolished until atropine 200 µg/kg had been given

retory responses to stimulation of the tympanic nerve and postganglionic nerves

eemed that all the glandular cells could be activated via the tympanic nerve au e it was possible by means of electrical stimulation of the nerve to attain the hest secretory rate which could be induced by reflex stimulation (Fig 2 continu line) Excitation of the tympanic nerve in the present report was about as ctive as the simultaneous stimulation in a previous report (Holmberg 1971) of h the auriculo-temporal nerve and the nerves on the internal maxillary artery g 2 dashed line) This finding together with the observation that reflexly in ed ecretion is very slow or abolished after division of the two latter sets of ves (Holmberg 1971) further supports the view that the overwhelming part of secretory cells are activated by impulses which travel in the tympanic nerve l which are then conveyed via the auriculo-temporal nerve and the fibres on the ernal maxillary artery to the gland

nervation experiments

er division of the tympanic nerve reflexly induced secretion was found to be ally abolished in 5 glands out of 10 whereas 3 3 5 6 12 % of the preoperative rate could be induced in the others These responses were totally abolished the injection of atropine 200 µg/kg intracardially and were thus mediated by olinergic nerves Possibly the magnitude of the remaining secretion was somewhat reased by denervation supersensitivity of the glandular cells because the responses the operated glands to methacholine 1 µg/kg intracardially where 5—10 times large as those of the unoperated control glands

The reduction of reflexly induced secretion was due to the division of the tympanic rve and not to any other consequence of the operation because in the ham erated animal reflexly induced ecretory rate was found to persist undiminished three weeks

Discussion

can be concluded from the present experiments that the overwhelming part of d in some animals probably all the preganglionic parasympathetic secretory nerves the dog s parotid travel via the tympanic nerve The secretory impul es are then nveyed to the gland via the auriculo-temporal nerve and the nerves on the internal axillary artery predominantly but also to a slight extent via the facial nerve and ssibly other nerves At least in some dogs there is a secretory pathway outside the mpanic nerve but it eems to contain only very few secretory fibres and its course mains unknown.

Earlier attempts have been made to trace secretory nerves to the dog's parotid outside the tympanic auriculo temporal and facial nerves and the internal maxillary artery (Holmberg 1971) Many cranial nerves were then electrically stimulated and special attention was paid to the chorda tympani which seems to convey secretory impulses to the human parotid gland (Reichert and Poth 1933 Diamant and Wiberg 1965) but the search was fruitless and was not repeated in the present experiments

The existence in the dog of such unknown nerves outside the tympanic one was suggested by the finding of Andreyev and Podkopayev (1928) that reflexly induced secretion was reduced only by 75—80 % when the tympanic nerve had been divided Their results are in conflict with those of Loeb (1869) who reported complete abolishment of reflexly elicited secretion after the same operation The present finding with complete abolishment in half the number of glands studied and 12 % or less remaining in the others indicate that the tympanic nerve is the most important preganglionic secretory nerve to the canine parotid but that there might be other pathways too at least in some dogs

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In Vitro Work Load and Rat Heart Metabolism

I Effect on protein synthesis

By

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Abstract

Hjalmarson Å and O Isaksson *In vitro work load and rat heart metabolism I Effect on protein synthesis* Acta physiol scand 1972 *86* 126—144

The isolated working rat heart preparation was used to study the effects of well defined work loads on heart protein synthesis The rate of protein synthesis was evaluated by measuring the incorporation rate of phenylalanine ^{3}H into whole heart protein Increased pressure load (afterload) accelerated the protein synthesis A stimulatory effect was demonstrated with glucose or palmitate as substrate and with 1 or 5 times the normal rat plasma levels of all amino acids in the perfusion medium The protein synthesis of both control and loaded hearts was significantly accelerated with palmitate as substrate or with high levels of all amino acids The work load effect could thus be obtained under conditions optimal for heart protein synthesis *in vitro* In contrast to the stimulatory effect of increased afterload the rate of protein synthesis was not changed when the left atrial filling pressure (preload) was increased although the external heart work was raised several times End systolic volume was markedly increased in pressure overloaded hearts compared to hearts perfused under control conditions or with increased preload It is concluded that an increase in contractile tension during systole and/or an increase in the end systolic volume was a necessary requirement for the acceleration of protein synthesis

The heart like most organs of the body will increase in size in response to increased physiological demand Heart hypertrophy is thought to be an important adaptive mechanism whereby the heart is able to sustain an increased work load (Meerson 1969) Although compensatory hypertrophy represent general cellular responses very little is known at present of the biochemical mechanisms by which physiological demand determines cell size Much uncertainty still prevails also with regard to the changes in cardiac mechanics initiating cardiac enlargement In hypertrophied hearts there is an increase in protein content and in the average diameter of myofibril which together with absence of mitotic figures has led to the suggestion that the increase in myocardial mass is due to an enlargement of cells rather than cell division (Linzbach 1960 Sumner and McIntosh 1963 Meerson 1969) From studies of ^{14}C amino acid incorporation into heart protein *in vivo* it has been concluded that the

increased protein content of the hypertrophied heart is the result of an elevated rate of protein synthesis (Cohen *et al* 1966 Zühlke *et al* 1966) Recent evidence suggests that activity of the ribosome cycle is an important factor in controlling the rate of protein synthesis in the heart (Morgan *et al* 1971 a b Jefferson *et al* 1971)

Cardiac hypertrophy has generally been studied *in vivo* as an adaption to a prolonged increase in work load for the heart This does not permit a definition of the specific mechanical stimuli which induce hypertrophy The advantage of an isolated working heart preparation is that the mechanical variables can be closely controlled and the specific mechanical stresses which induce specific changes in myocardial metabolism can be defined Schreiber and co-workers (Schreiber *et al* 1966 1967 a b 1968 1969) have performed studies on cardiac protein synthesis in the isolated perfused heart in which they were able to demonstrate an increased rate of protein synthesis by cardiac overload *in vitro* However in these *in vitro* studies the work load was not well characterized and the hearts defined as controls developed pressures below the physiological range

The control mechanisms for protein synthesis in mammalian systems are not well defined There are several steps which are considered to constitute possible control sites in the synthesis of protein (a) amino acid tranport (b) intracellular activation of amino acids (c) transcription processes and (d) translation of messenger RNA at the ribosomes In the present investigation the effects of well defined work loads on the incorporation of phenylalanine ^{3}H into protein of the isolated working rat heart was investigated In two succeeding papers the effects of increased work load on amino acid transport and on ribosomal aggregation will be presented

Methods

Animals

Fed female rats of the Sprague Dawley strain weighing 200—240 g maintained on a standard pellet diet (Type 213 Anticimex Stockholm) and water *ad libitum* were used

Radioactive materials

L phenylalanine ^{3}H (G) and sucrose ^{14}C (U) were obtained from the New England Nuclear Corp Boston USA The substances were used with the following specific activities L-phenylalanine ^{3}H (G) 1 25 μCi/μmole sucrose ^{14}C (U) 3 76 Ci/mole 0 1 μCi of phenylalanine ^{3}H per ml perfusion medium was used when the perfusion medium contained all amino acids at normal plasma levels and 0 5 μCi/ml perfusion medium when 5 times the normal levels of amino acids was used Sucrose-^{14}C was added to the perfusion medium to make a molarity of 0 01 mM

Perfusion medium

The perfusion medium was Krebs Henseleit bicarbonate buffer (Krebs and Henseleit 1932) pH 7 40 containing the disodium salt of EDTA (0 5 mM) and glucose (14 mM) or palmitate (1 5 mM) All amino acids at normal rat plasma levels were employed to simulate the true rat serum concentrations (in some experiments 5 times the normal plasma levels) The following concentrations (mM × 10) were used aspartic acid 4 7 asparagine 7 4 glutamic acid 11 6 glutamine 30 5 serine 17 6 threonine 21 8 glycine 26 5 alanine 37 7 methionine 5 0 valine 19 0 isoleucine 9 1 leucine 13 1 phenylalanine 7 7 tyrosine 6 0 lysine 24 2 histidine 7 8 arginine 6 2 proline 15 8 cysteine 6 8 tryptophan 10 0 (Morgan *et al* 1971 a)

When palmitate was used as substrate the fatty acid albumin complex was prepared in the following way Palmitic acid (99 % Lot no 128 B-0450 Sigma) was dissolved in 50 % ethanol containing potassium carbonate The ethanol was evaporated and the resulting warm dissolved potassium soap was aspirated by a syringe and injected into a 15 % albumin solution (F

V from bovine plasma Batch SD 0370 Armour England) The resulting fatty acid albumin solution was dialyzed overnight against 8—10 volumes of Krebs Henseleit bicarbonate buffer On the day of perfusion the fatty acid albumin solution was diluted to a final albumin concentration of 3 % in buffer containing all additional components of the final medium.

Perfusion technique

Rats were anesthetized with Nembutal (60 mg/kg b w) and the rapidly excised hearts were transferred into ice chilled saline The aorta and the left atrium were cannulated and retrograde perfusion with Krebs Henseleit bicarbonate buffer at 37 C from a reservoir 70 cm above the heart was started immediately The time period from the excision of the heart to the start of the retrograde perfusion was less than 30 s Following a 5 min retrograde washout period the heart was either perfused as a modified Langendorff preparation (retrograde perfusion non working Neely *et al* 1967 a) or as a working heart preparation (anterograde perfusion working Morgan *et al* 1965 Neely *et al* 1967 a) The recirculating volume (40 ml) was continuously gassed with 95 % O_2—5 % CO_2 equilibrated with water at 37 C All perfusions were carried out at 37 C

The perfusion pressure and heart work in the Langendorff preparation could be changed by varying the degree of compression of the plastic tubings by the peristaltic pump In the working preparation cardiac work could be increased by elevating the left atrial filling pressure (increased volume load increased preload) or by increasing the resistance to the outflow of the left ventricle (increased pressure load increased afterload) To provide some elasticity to the rigid aortic tubings a sidearm of a plastic tube filled with 2 5 ml of air was always connected to the lowest part of the aortic tube This artificial Windkessel markedly influenced the work and coronary flow of the anterogradely perfused heart

Under standard conditions (working preparation referred to as control in the text) the left ventricle pumped against a hydrostatic pressure head of 70 cm H_2O with a left atrial filling pressure of 10 cm H O Hearts perfused under these conditions were considered controls for subsequent studies since these hearts showed stable performance up to 3 h A small amount (about 20 %) of the glucose taken up was converted to lactic acid and the glycogen content was well maintained indicating good oxygenation Pressure overload was accomplished by total constriction of the aortic tube For both control and overloaded hearts optimal oxygenation was maintained by an overflow system At the end of perfusion the heart was cut in beaker containing ice-chilled 0 9 % NaCl Immediately thereafter the heart was opened and a section of about 200 mg of the lower lateral wall of the left ventricle was rinsed rapidly in rebs Henseleit bicarbonate buffer blotted on filter paper and weighed on a torsion balance

Determination of heart volumes

The exterior diameter of the heart was measured by colour motion picture recordings (64 exposures/s at a magnification of × 2 at different preloads and afterloads The exposure with the largest exterior heart diameter in each heart cycle was considered to represent the end diastolic exterior heart diameter A millimeter scale placed in the focus plane was used for calibration

In the same experiment 5 hearts were rapidly excised from anesthetized rats of equal size and dropped into ice chilled saline The aorta was cannulated to a syringe (1 ml) and the aorta ligated below the openings of the coronary arteries Ice cold saline was injected with the syringe in sufficient amounts to increase the heart diameter to fill the space between a pair of calipers pre set at various distances In principle the same ventricle volume heart diameter relationship was obtained by motion picture recording

Measurements of performance of heart

Heart rate aortic pressures coronary flow and cardiac output were monitored at 30 min intervals For recording aortic pressures a transducer was attached to a manifold of the aortic tube and the pressure tracings were registered by a Sanborn recorder model 16—1300 S Coronary flow was measured by collecting the fluid dropping from the heart and cardiac output (working preparation) was estimated by adding the coronary flow to the aortic outflow Heart rates were determined from pressure recordings

Measurements of glycogen content glucose uptake and lactic acid production

For the determination of glycogen the hearts were frozen between blocks of aluminium cooled to the temperature of liquid nitrogen (Wollenberger *et al* 1960) while the heart was still being perfused Approximately 50 mg of the frozen tissue was boiled in 0 4 ml of 30 % KOH for 15 min to extract the glycogen The glycogen was precipitated with 0 ? ml of Na_2SO_4 (8 %) and 1 ? ml of ethanol (96 %) and was thereafter enzymatically split into glucose by

TABLE I Total tissue water of isolated perfused rat heart

Addition to perfusate		Perfusion time (min)						
		15		60		90		120
		Non working						
Glucose 14 mM	A					79.9 ± 0.22 (6)	p < 0.05	
	B	80.6 ± 0.32 (4)		80.5 ± 0.26 (4)		81.0 ± 0.28 (6)		
		Working						
Glucose 14 mM	(−)	80.9 ± 0.41 (4)	p < 0.05	81.6 ± 0.38 (4)	p < 0.05	81.3 ± 0.27 (6)	p < 0.05	81.3 ± 0.27 (6)
	(+)	82.7 ± 0.60 (4)		83.8 ± 0.51 (4)		83.5 ± 0.30 (6)		83.9 ± 0.47 (6)
Albumin (3 %) palmitate 1.5 mM	(−)	80.9 ± 0.21 (4)	NS	79.5 ± 0.45 (4)	p < 0.05			
	(+)	81.3 ± 0.28 (4)		81.8 ± 0.36 (4)				

All hearts were preperfused retrogradely for 5 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels. The hearts were then perfused retrogradely (non working) or anterogradely (working) for various periods with the same buffer containing glucose or palmitate as substrate. The perfusion pressure in the non working preparation was 30 (A) and 60 (B) mm Hg. The working rat hearts were perfused with (pressure overload +) and without (control −) total aortic constriction at 10 cm H_2O left atrial filling pressure. Values are means ± S.E. of per cent of wet tissue weight. Number of hearts in each group is indicated in parentheses.

α 1,4-α 1,6 amylase and the glucose was finally assayed by the glucose oxidase method. The glycogen content is expressed as micromoles of glucose equivalent per gram dry weight. Glycogen from rabbit liver was used as a standard.

The glucose uptake of the heart was determined by measuring the initial and final glucose concentration of the perfusion media with the glucose oxidase method (Saifer and Gerstenfeld 1958). The uptake of the glucose is expressed as μmol/g dry weight × h. Lactic acid production of the heart was calculated from the amount of lactic acid found in the perfusion medium at the end of the perfusion period and was determined by an enzymatic method as described by Lundholm and co-workers (Lundholm *et al.* 1963). The lactic acid production is expressed as μmol/g dry w. × h.

Determination of incorporated radioactivity into protein

After homogenizing in 5 % PCA (perchloric acid) the precipitate was spun down and washed once with 5 % PCA. Excess of non labelled amino acid was added. The precipitate was then heated for 15 min in a boiling water bath and washed twice with the same solution. The insoluble material was extracted with 4 ml of 0.3 N NaOH and precipitated with 1 ml of 6 N *HCl*. The precipitate was then washed twice with 1 % potassium acetate in ethanol and thereafter 3 times with a mixture of ether ethanol chloroform (1 3 2) and finally twice in ether and dried (Munro and Fleck 1969). The purified protein was dissolved in 2 ml of 1 N NaOH and was heated in a boiling water bath for 25 min. Samples were taken for protein determination according to Lowry *et al.* (1951) with bovine serum albumin as a standard (Sigma, stock 905 10). For radioactivity measurements 50 μl of the same solution of proteins was added to 0.5 ml soluene—TM 100 (Packard). Liquid scintillation counting was carried out with 10 ml of a solution consisting of 90 % PPO (2,5-diphenyloxazole) and 10 % Bis—MSB [*p*-Bis (0 methylstyryl) benzene] (Permablend TM III Packard) in toluene (5.5 g/1000 ml) in a Packard Model 3375 liquid scintillation spectrometer with a counting efficiency of 32—34 for ^{3}H. Duplicate samples were always counted. The degree of quenching was measured with automatic external standardization and DPM (disintegration per minute) values for individual samples were calculated. The incorporation of radioactivity into protein is expressed as DPM/mg protein × 10^{3}

TABLE II Sucrose ^{14}C spaces of isolated perfused rat heart

Addition to perfusate		Perfusion time (min) 15		60		90		120	
		Non working							
Glucose 14 mM	A					30 0±0 6 (6)			
	B	32 8±0 5 (4)		33 0±0 8 (8)		37 6±0 6 (6)^a			
		Working							
Glucose 14 mM	(−)	35 1± 1 0 (4)	N S	35 9± 1 3 (4)	p < 0 05	36 4± 0 7 (6)^b	p < 0 05	35 6± 0 8 (6)	p < 0 05
	(+)	37 1± 1 4 (4)		43 0± 1 2 (4)		44 7± 0 9 (6)		44 8± 1 0 (6)	
Albumin (3) palmitate 1 5 mM	(−)	33 4± 0 9 (4)	N S	34 2± 0 6 (4)	p < 0 05				
	(+)	36 0± 0 7 (4)		39 4± 1 0 (4)					

All hearts were preperfused retrogradely for 5 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels The hearts were then perfused retrogradely (non working) or anterogradely (working) for various periods with the same buffer containing sucrose C and glucose or palmitate as substrate The perfusion pressure in the non working preparation was 30 (A) and 60 (B) mm Hg The working rat hearts were perfused with (pressure overload +) and without (control −) total aortic constriction at 10 cm H_2O left atrial filling pressure Values are means ±S E of per cent of wet tissue weight Number of hearts in each groups is indicated in parentheses a vs b p < 0 05

Determination of total tissue water and extracellular water

Total tissue water was determined by drying a piece of the lower part of the lateral wall of the left ventricle to constant weight at 100 C in a vacuum oven The values are expressed as per cent of the wet tissue weight (ml/100 g tissue) From this procedure the wet dry weight ratio was determined

For the estimation of the phenylalanine ^{3}H distribution ratio it was necessary to determine the water content of the heart and the volumes of the extra and intracellular compartments after different perfusion periods and under various experimental conditions Table I shows that the total tissue water was stable with time and that no difference existed between non working and working hearts perfused under the control conditions Total tissue water was significantly increased by pressure overload both when the hearts were perfused with glucose or with albumin (3 %) —palmitate as substrate but the increase with albumin—palmitate was of small magnitude

The extracellular space of the perfused heart was determined by measurements of the distribution of sucrose ^{14}C between the tissue and the perfusate The sucrose ^{14}C space is expressed as per cent of the total wet tissue weight (ml/100 g tissue) Table II shows that the sucrose-^{14}C space did not increase with time in the non working and working hearts perfused under the control conditions The sucrose ^{14}C space was somewhat increased in working hearts perfused under the control conditions compared to non working hearts The sucrose ^{14}C space was significantly increased by pressure overload both in hearts perfused with glucose or with albumin (3 %)—palmitate The increase in sucrose ^{14}C space by cardiac overload was smaller in hearts perfused with albumin—palmitate Taken together these data show that the whole increase in the total tissue water by pressure overload could be accounted to the increase in the extracellular space (determined with sucrose ^{14}C)

TABLE III Mechanical performance of working rat heart preparation

Parameter	Duration of perfusion min							
	30		60		90		120	
	Control (5)	Pressure overload (5)	Control	Pressure overload	Control	Pressure overload	Control	Pressure overload
Peak systolic pressure mm Hg	96 ± 2	160 ± 4	98 ± 4	158 ± 6	98 ± 2	152 ± 8	94 ± 3	140 ± 4
Diastolic pressure mm Hg	44 ± 3	120 ± 4	47 ± 2	120 ± 6	46 ± 2	118 ± 3	48 ± 2	110 ± 2
Heart rate beats/min	244 ± 15	255 ± 11	238 ± 18	248 ± 16	240 ± 15	225 ± 14	236 ± 18	234 ± 14
Coronary flow ml/g d w x min	78 ± 5	200 ± 10	78 ± 6	202 ± 10	3 ± 4	182 ± 14	73 ± 4	150 ± 18
Cardiac output ml/g d w x min	275 ± 14	200 ± 10	290 ± 24	202 ± 10	254 ± 16	182 ± 14	235 ± 15	150 ± 18

Hearts were perfused anterogradely (working) for 120 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels Performance measurements were carried out at 30 60 90 and 120 min as described in Methods *The pressure overload was accomplished by total constriction of the aortic tube The left atrial filling pressure in* both groups was 10 cm H_2O Values are means ± S E Five hearts in each group were perfused

Distribution ratio of phenylalanine-³H

The accumulation of labelled amino acid in the heart muscle is expressed as *distribution ratio* (counts/min per ml intracellular water counts/min per ml perfusate) It is assumed that the concentration of labelled amino acid in the extracellular water at the end of the perfusion was the same as in the perfusate The concentration of labelled amino acid in the intracellular water was calculated by subtracting the amount of radioactivity in the extracellular space (determined with sucrose-^{14}C) from the radioactivity in the total tissue water The degree of quenching was tested by automatic external standardization in each sample and it was found to be the same for the tissue extracts and their respective perfusion media It was therefore not necessary to correct the counting data for quenching prior to the calculations of the extracellular spaces and the distribution ratios

Statistics

Analysis of variance (with one criterion of classification) followed by the Student Newman Keuls multiple range test was used (Woolf 1968) A p-value of 0 05 or less was considered significant in this study

Results

Mechanical performance of the isolated working rat heart

Table III shows performance and stability of the working heart preparation perfused with and without pressure overload (total aortic constriction) at a left atrial filling pressure of 10 cm H_2O Peak systolic pressure increased from approx 95 mm Hg in the control hearts to approx 160 mm Hg in the overloaded hearts with a simultaneous increase in coronary flow from about 75 to about 200 ml/g d w x min As can be seen from Table III the hearts in the control group showed stable per

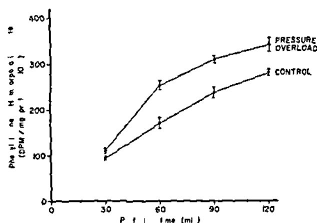


Fig 1 Effects of increased pressure load on incorporation of phenylalanine-^3H into protein of perfused rat heart Hearts were perfused for various periods with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels at a left atrial filling pressure of 10 cm H_2O with (pressure overload) and without (control) total constriction of the aortic tube Each point represents mean of 6—14 observations and standard errors are indicated by vertical lines The effects of pressure overload are significant ($p < 0.05$) at all time periods studied

formance for 120 min A slight deterioration of mechanical function was observed in the overloaded hearts after a perfusion period of 90 min as judged by a decrease in aortic pressures and coronary flow Heart rates did not change significantly with time in any of the groups

Effects of increased pressure work load in incorporation of phenylalanine-^3H into heart protein

Fig 1 shows a time course study of the effects of increased pressure load on the incorporation of phenylalanine-^3H into heart protein The hearts were perfused for 30—120 min The overload stimulated the incorporation of phenylalanine at all perfusion periods studied ($p < 0.05$) During the 120 min perfusion it can be seen from the figure that the phenylalanine-^3H incorporation rate declined with time The incorporation for both control and overloaded hearts was significantly higher during the first than during the second hr

Work load *in vivo* and *in vitro* cannot be directly compared due to *e g* the very different metabolic and hemodynamic conditions The present definition of control and overload work level *in vitro* might not represent normal and overloaded hearts *in vivo* It seemed therefore necessary to study different levels of afterload on the incorporation of phenylalanine in the working rat heart as shown by Table IV The table shows that a positive relationship between the afterload and the incorporation rate of phenylalanine exists in the working heart The glycogen content was not affected by the different work levels but the lactic acid production was significantly elevated in the group with the lowest afterload compared to the other groups This in combination with the decreased phenylalanine incorporation may indicate that the coronary flow was insufficient for the energy demands of the hearts in this group The distribution ratio of phenylalanine-^3H did not show any greater changes in this or in the successive experiments but a difference was seen between the groups with the highest and lowest afterload indicating that the membrane transport and incorporation of phenylalanine proceeded at different rates

TABLE IV Effects of increased pressure work load on incorporation of phenylalanine H into heart protein phenylalanine H uptake final glycogen content lactic acid production and mechanical performance of the isolated working rat heart

Parameter	Hydrostatic pressure against the left ventricle cm H O			
	40 (6)	70 (10)	70 partial constriction *of the aortic* tube (4)	Total constriction *of the aortic* tube (10)
Phenylalanine H incorporation DPM/mg protein × 10^{-3}	195±10[a]	235±9[b]	267±6	376±13[d]
Phenylalanine H distribution ratio	1 41±0 03	1 31±0 03[f]	1 35±0 04	1 25±0 04[h]
Final glycogen content μmol/g d w	77±5	68±6	–	70±8
Lactic acid production μmol/g d w × h	450±15[i]	105±16	140±15	244±46[j]
Coronary flow ml g d w × min	28±4	87±6	135±8	238±21
Peak systolic pressure mm Hg	48±4	94±3	118±5	164±8
Diastolic pressure mm Hg	16±3	47±2	78±3	170±4

Hearts were perfused anterogradely (working) for 90 min at different hydrostatic pressures against the left ventricle The perfusion medium was Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels Left atrial filling pressure was 10 cm H O Values are means±S E Number of hearts in each group is indicated in parentheses a vs b c d $p < 0.05$ b c vs d $p < 0.05$ e vs f h $p < 0.05$ i vs j $p < 0.05$

TABLE V Effects of increased pressure work load on incorporation of phenylalanine H into heart protein and phenylalanine H uptake of isolated working rat heart perfused with various amino acid concentrations with glucose or palmitate as substrate

Addition to perfusate	Experiment 1 condition	Phenylalanine H incorporation rate DPM/mg protein × 10⁻		Phenylalanine H distribution ratio	
1 × AA glucose 14 mM	Control	279± 7 (13)	$p < 0.05$	1 37±0 04[i]	
	Pressure overload	335±12 (13)[b]		1 32±0 04	
5 × AA glucose 14 mM	Control	384±22 (5)	$p < 0.05$	1 06±0 0 [i]	NS
	Pressure overload	489±32 (5)[d]		0 93±0 01	
1 × AA albumin (3) palmitate 1 5 mM	Control	315± 6 (14)	$p < 0.05$	1 30±0 04	$p < 0.05$
	Pressure overload	353± 4 (13)[f]		1 07±0 07	
5 × AA albumin (3) palmitate 1 5 mM	Control	440±20 (5)[e]	p 0 05	0 90±0 04[k]	
	Pressure overload	575±20 (5)[h]		0 94±0 15	

Hearts were perfused anterogradely (working) for 120 min with Krebs-Henseleit bicarbonate buffer containing various amino acid concentrations and with glucose or palmitate as substrate as indicated by the table 1 × AA = all amino acids at normal plasma levels 5 × AA = all amino acids at 5× normal plasma levels To obtain the same specific activity in the perfusate with 5 × AA as in the perfusate with 1 × AA 5 times the normal amount of radioactivity was supplied to the former group Values are means±S E Number of hearts in each group is indicated in parentheses a vs e $p < 0.05$ c vs e $p < 0.05$ c vs g $p < 0.05$ d vs h NS i vs j k $p < 0.05$ j vs k NS

TABLE VI. Effects of increased pressure work load on incorporation of phenylalanine-^3H into heart protein after normalization of pressure load on the heart

Perfusion period 0 to 60 min: Experimental condition	Coronary flow ml/g d. w. × × min	60 to 120 min: Experimental condition	Coronary flow ml/g d. w. × × min	Phenylalanine-^3H incorporation DPM/mg protein × 10^{-4}	Phenylalanine-^3H distribution ratio
Control (12)	90 ± 5	Control	86 ± 6	158 ± 11	1.29 ± 0.03
Pressure overload (11)	226 ± 18	Control	88 ± 7	248 ± 18	1.37 ± 0.07

Hearts were perfused anterogradely (working) for 120 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels. A tracer dose of phenylalanine-^3H (spec. activity 8.3 Ci/mmole) was added to the recirculating medium after an initial perfusion period of 60 min with and without aortic constriction. The overload was accomplished by total constriction of the aortic tube. The left atrial filling pressure was 10 cm H_2O. Values are means ± S.E. Number of hearts in each groups is indicated in parentheses.

Changing the amino acid concentration of the perfusate from the normal rat plasma amino acid level to 5 times the normal level increases the incorporation of phenylalanine into heart protein (Morgan 1971 a). Perfusion of the heart with palmitate as substrate instead of glucose also increases the incorporation rate (Rannels *et al.* 1970). It was considered important to study the effects of work load at different basal rates of protein synthesis. Table V shows that pressure overload increased the incorporation of phenylalanine both with palmitate as substrate and when the buffer solution was fortified with amino acids 5 times the normal rat plasma level. It can also be seen from the table that an increase in perfusate amino acid concentrations stimulated the incorporation of phenylalanine in all the groups studied whereas a decrease in the distribution ratio of phenylalanine was demonstrated in these groups. It is further seen that palmitate stimulated the control incorporation of phenylalanine but to a smaller magnitude than when the amino acid levels were increased 5 times.

As shown by Table IV the coronary flow increased markedly when the afterload was elevated. One explanation to the stimulated incorporation of phenylalanine during this experimental condition could be the better supply of substrates and oxygen to the hearts with higher coronary flows. To test this possibility the incorporation of phenylalanine was studied during a perfusion period of 60 min after a preceding perfusion period of 60 min with and without total aortic constriction as shown by Table VI. It can be seen from this table that the incorporation rate of phenylalanine was significantly increased in the hearts that were overloaded during the first 60 min although there was no difference in coronary flow between the two groups during the second perfusion period. This indicates that the coronary flow

TABLE VII Effects of increased left atrial filling pressure on incorporation of phenylalanine-^{3}H, phenylalanine-^{3}H uptake, final glycogen content, glucose uptake, lactic acid production and mechanical performance of isolated working rat heart

Parameter	Left atrial filling pressure, cm H_2O		
	2	7	15
Phenylalanine-^{3}H incorporation DPM/mg protein × 10^{-3}	294 ± 13	275 ± 17	294 ± 13
Phenylalanine-^{3}H distribution ratio	1.25 ± 0.03	1.33 ± 0.04	1.37 ± 0.11
Final glycogen content, µmol/g d. w.	74 ± 8	65 ± 5	72 ± 7
Glucose uptake, µmol/g d. w. × h	118 ± 16	194 ± 36	269 ± 21
Lactic acid production, µmol/g d. w. × h	76 ± 5	96 ± 14	126 ± 36
Peak systolic pressure, mm Hg	68 ± 1	89 ± 3	107 ± 3
Diastolic pressure, mm Hg	49 ± 1	44 ± 2	37 ± 3
Coronary flow, ml/g d. w. × min	73 ± 3	77 ± 3	89 ± 5^b
Cardiac output, ml/g d. w. × min	112 ± 20	280 ± 21	360 ± 42

Hearts were perfused anterogradely (working) at various left atrial filling pressures with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels for 120 min. Values are means ± S.E. Nine hearts in each group were perfused. This experiment was repeated 3 times with similar results. a vs b: $p < 0.05$.

per se is not a limiting factor for the protein synthesis during this experimental condition. The data also show that the effect of overload on phenylalanine incorporation is sustained for some time after normalization of the cardiac work.

Effects of increased volume load on the incorporation of phenylalanine-^{3}H into heart protein

Another way to increase the heart work of the anterogradely perfused rat heart is to elevate the left atrial filling pressure. Table VII shows that the incorporation of phenylalanine was not significantly affected when the left atrial filling pressure was increased from 2 to 15 cm H_2O. The glucose uptake was increased by about 100 % and the cardiac output was increased about 3 times by this increase in preload. With a constant hydrostatic pressure head above the left ventricle and a low resistance to the aortic outflow, the mean aortic pressure is not changed significantly when the preload is increased. This is the main reason why the coronary flow was not further increased when the left atrial filling pressure was elevated. The relatively low coronary flow in the hearts perfused with high preload made it desirable to study the effects of different left atrial filling pressures at an elevated aortic diastolic pressure. Such an experiment is shown by Table VIII, in which the aortic diastolic pressure was held constant by varying the degree of aortic constriction. The incorporation rate of phenylalanine was not significantly affected by different left atrial filling pressures under this experimental condition, although the incorporation level was elevated compared to the foregoing experiment, probably due to the slight increase in afterload by this procedure. It is concluded from the experiments shown in Table VII and VIII that increased volume work (preload) does not significantly affect the incorporation of phenylalanine.

TABLE VIII Effects of increased left atrial filling pressure at a constant aortic diastolic pressure (80 mm Hg) on incorporation of phenylalanine ³H into heart protein uptake of phenylalanine ³H lactic acid production and coronary flow of isolated working rat heart

Parameter	Left atrial filling pressure cm H O		
	2	7	15
Phenylalanine ³H incorporation			
DPM/mg protein × 10 ¹	330±14	372±24	336±14
Phenylalanine ³H distribution ratio	1 21±0 05	1 33±0 05	1 24±0 03
Lactic acid production µmol/g d w × h	60±15	200±40	260±28
Coronary flow ml/g d w × min	96± 8	132±11	145± 6

Hearts were perfused anterogradely for 120 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels at a constant aortic diastolic pressure of 80 mm Hg A constant aortic diastolic pressure was maintained by partial and varied constriction of the aortic tube Values are means±S E 5 hearts in each group were perfused

Effects of increased perfusion pressure on the incorporation of phenylalanine ³H in the retrogradely perfused heart

When the perfusion pressure was increased from 60 to 120 mm Hg there was a small but significant increase in the incorporation of phenylalanine as shown by Table IX The increase in perfusion pressure also reduced the glycogen content and stimulated the lactic acid production indicating that the heart work was increased When the perfusion pressure was decreased from 60 to 40 or 30 mm Hg the incorporation did not decrease significantly but the glycogen content decreased and the lactic acid production increased indicating insufficient flow through the coronary arteries

To further investigate the mechanism by which the increase in perfusion pressure stimulated the incorporation of phenylalanine the left ventricle was perforated by a stab wound at the apex and was cannulated with a plastic tube (2 mm internal diameter) This minimized the tension to the ventricle wall during systole Table X

TABLE IX Effects of increased perfusion pressure on the incorporat on of phenylalanine H into heart protein pheynylalanine H uptake final glycogen content lactic acid production and coronary flow of the retrogradely (non working) perfused rat heart

Parameter	Perfusion pressure mm Hg			
	30	40	60	120
Phenylalanine H incorporation				
DPM/mg protein × 10	228±6	246±13[b]	243±6	278±7[a]
Phenylalanine H distribution ratio	1 26±0 01	1 32±0 04	1 ^9±0 02	1 ?5±0 03
Final glycogen content µmol/g d w	73±6		101±7[f]	68±5[f]
Lactic acid production µmol/g d × h	140±15		28±14	235±54
Coronary flow ml/g d w min	49±6	56±4	78±5	218±14

Hearts were perfused retrogradely (non working) for 90 min at various perfusion pressures with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels Values are means±S E 6 hearts in each group were perfused
a b c vs d p < 0 0ↄ f vs e g p ‹ 0 05

TABLE X Effects of increased perfusion pressure on the incorporation of phenylalanine H into heart protein phenylalanine H uptake and coronary flow of the retrogradely (non working) perfused heart with and without a perforated left ventricle

Perfusion pressure mm Hg	Number of experiments	Number of hearts	Phenylalanine H incorporation rate DPM/mg protein × 10⁻		Phenylalanine H distribution ratio	Coronary flow ml g d w × min
60	3	12	241 ± 7	p < 0 05	1 24 ± 0 02	78 ± 9
120	3	11	272 ± 6		1 28 ± 0 04	143 ± 16
60 left ventricle perforated	3	12	231 ± 9	N.S	1 28 ± 0 04	85 ± 6
120 left ventricle perforated	3	12	235 ± 17		1.24 ± 0 07	163 ± 12

Hearts were perfused retrogradely for 90 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels with and without a perforated left ventricle The left ventricle was perforated by a puncture wound in the apex Values are means ± S E

hows that no significant difference in the incorporation rate of phenylalanine could be demonstrated when the perfusion pressure was increased from 60 to 120 mm Hg when the left ventricle was perforated in contrast to the small difference in incorporation rate that was found in hearts without perforation 3—10 ml of perfusate/heart × min dropped out from the plastic tube in the perforated hearts indicating leakage through the aortic valves or flow to the left ventricle from the Thebesian veins The experimental data in Table X further strengthen the hypothesis that the coronary flow *per se* is of less importance in regulating the protein synthesis in the perfused heart when the left ventricle does not perform external pressure work

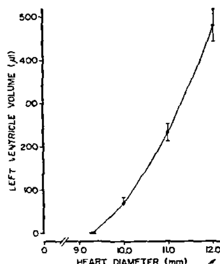


Fig 2 Relation between external heart diameter and left intraventricular volume Five hearts (rat weight 270 g) were excised from anesthetized rats and dropped into ice-chilled saline The aorta was cannulated to a syringe (1 cc) containing ice-chilled saline and ligated below the openings of the coronary arteries Ice-cold saline was injected with the syringe in sufficient amounts to increase the heart diameter to fill the space between a pair of calipers pre set at various distances In principle the same ventricle volume heart diameter relationship was obtained by motion p c ture recording The values in the figure are means ± S E

TABLE XI Effects of different work loads on end diastolic heart diameter cardiac output heart rate left ventricle end diastolic volume stroke volume and systolic residual volume of isolated working rat heart

Parameter	Left atrial filling pressure cm H O			
	0	5	15	10 Total constriction of the aortic tube
End diastolic heart diameter mm	10 1−10 6	10 5−10 8	11 1−11 5	11 7−12 1
Cardiac output ml/min	11−14	24−43	50−62	28−35
Heart rate beats/min	220−260	230−265	245−270	230−270
Left ventricle end diastolic volume μl	85−162	145−195	255−350	400−505
Stroke volume μl	48−55	91−165	204−230	114−154
Systolic residual volume μl	29−97	5−104	51−1[illegible]8	284−375

Hearts (rat weight 220 g) were perfused anterogradely (working) with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) End diastolic heart diameter was measured at different left atrial filling and perfusion pressures as described in Methods Stroke volume for individual hearts was calculated by dividing cardiac output with heart rate Left ventricle end diastolic volume was estimated by the left ventricle volume heart diameter plot as shown by Fig 2 Systolic residual volume was calculated by subtracting stroke volume from left ventricle end diastolic volume Range of measured and calculated values for 5 hearts are given

The data presented above indicates that an increased coronary flow *per se* does not stimulate the incorporation of phenylalanine under the present conditions To further analyse the difference between increased preload and increased afterload concerning the incorporation rate of phenylalanine, a heart diameter—left ventricle volume curve was plotted as shown by Fig 2 (for experimental details see Methods) By measuring the end diastolic heart diameter at different preloads and when the aortic tube was totally constricted it was possible to get an average estimate of the left ventricle end diastolic volume and the systolic residual volume as shown by Table XI The table shows that the left ventricle end diastolic volume increased when the left atrial filling pressure was elevated but the systolic residual volume was not significantly affected by increased preload When the afterload increased the end diastolic volume increased further but the most striking difference between hearts with increased preload and hearts with increased afterload was the marked increase in systolic residual volume in pressure overloaded hearts This indicates that the increased afterload was of considerable stress to the left ventricle and as a consequence the fiber length was increased both during diastole and systole

Discussion

By elucidating rate limiting factors in cardiac protein synthesis under different experimental conditions insight may be gained into control mechanisms of protein synthesis Previous investigations *in vitro* and *in vivo* indicate that cardiac protein

synthesis is regulated by several factors including hormones Important hormones for regulation of heart protein synthesis are thyroxine (Scow 1959 Cohen *et al* 1966 Cohen and Whitbeck 1969) growth hormone (Earl and Korner 1966 Ahren *et al* 1969 Hjalmarson *et al* 1970) and insulin (Wool 1965, Morgan *et al* 1971 b) Other important factors are the supply of substrates (Rannels *et al* 1970) adequate oxygenation (Jefferson *et al* 1971) amino acid supply (Morgan *et al* 1971 a) and the mechanical activity of the tissue (Schreiber *et al* 1966 Kako and Minelli 1969) Hypophysectomy has been shown to significantly reduce the rate of protein synthesis as well as the cardiac hypertrophy following aortic constriction (Beznak 1964, Morkin *et al* 1968 Hjalmarson *et al* 1970) A great number of *in vivo* studies have shown that increased cardiac work leads to accelerated cardiac protein synthesis and hypertrophy As mentioned in the Introduction this hypertrophy is considered to be an important compensatory mechanism whereby the tissue is able to sustain the increased work load From the *in vivo* techniques used to induce hypertrophy it is difficult to accurately assess the control mechanisms involved in the accelerated protein synthesis The majority of experimental studies concerning heart hypertrophy have been carried out *in vivo* which makes it difficult to establish correlations between various parameters of heart function and early changes in cardiac enlargement The perfused rat heart does not have these disadvantages By using this preparation a precise control of hemodynamic parameters is possible and secondary homeostatic mechanisms are excluded A further advantage is that the composition of the perfusion medium can be exactly controlled and altered which makes it possible to further delineate rate limiting factors

This study clearly demonstrates that an increased afterload stimulates protein synthesis in the isolated perfused heart after a perfusion period of only 30 min This is in accordance with previous *in vitro* studies using the isolated guinea pig heart (Schreiber *et al* 1966) or heart lung preparation from the rat (Kako and Minelli 1969) The main difference between this study and these previous is that near to normal work loads have been applied to the control hearts which has not been the case in earlier *in vitro* investigations and that the work loads have been accurately defined The overload in this study was accomplished by total constriction of the aortic tube which increased the aortic pressures to supraphysiological levels The pressure overload caused a significant oedema in the heart muscle but no marked decrease in muscle function was seen during 90 min of perfusion When perfusions were carried out for longer than 90 min periods there was a decrease in the function of some hearts However there was no drastic decrease in cardiac glycogen content or increase in lactic acid production after 120 min of perfusion in the overloaded hearts indicating unimpaired membrane transport of substrates and oxidative metabolism

Table IV shows that if the afterload of the hearts was diminished too much a significant decrease in protein synthesis was observed This could be due to a reduction of coronary flow in relation to heart work which was supported by the observation of a marked increase in lactic acid production under this condition This ob-

ervation is in accordance with previous studies with atrial muscle (Cohen *et al* 1969) It was demonstrated in these studies (Cohen *et al* 1969) that protein synthesis was decreased when oxygen tension was lowered or when the generation of high energy phosphates was inhibited In this connection it is pertinent to note that a stable mechanical performance *per se* does not acertain adequate cell function concerning protein synthesis Determination of lactate production and final glycogen content is required as a test for sufficient supply of oxygen On the other hand it can be questioned whether the increased coronary flow *per se* associated with the pressure overload could be the stimulatory factor for the increased phenylalanine incorporation The results of the present study strongly indicate that factors other than an increase in coronary flow are involved in the stimulation of heart protein synthesis

Neely *et al* (1967a) have shown that the retrogradely perfused heart develops tension equal to the aortic perfusion pressure In the present study no difference in incorporation rates was seen when the perfusion pressure was varied between 30 and 60 mm Hg but when the perfusion pressure was further increased to 120 mm Hg a significant stimulatory effect could be seen This effect was inconsistent and was only found in 3 expts out of 5 When the left ventricle in several experiments was perforated by a puncture wound in the apex no stimulation of incorporation was detectable as mentioned earlier As has been reported earlier (Neely *et al* 1967a) and also shown in this study there is a slow filling of the left ventricle in the retrogradely perfused heart This filling of the left ventricle may have arisen from the Thebesian veins or by leakage through the aortic valves This enables the retrogradely perfused heart to develop tension which should properly merit the non working heart another name as pointed out by Opie *et al* (1971) If it is postulated that increased contractile tension might be the trigger mechanism for the stimulation of protein synthesis with increased afterload the somewhat inconsistent results with the retrogradely perfused heart could be explained by different degrees of distortion of the aortic valves during the cannulation procedure

Increased preload was not found to stimulate protein synthesis although the external cardiac work was increased An increase in cardiac output without a change in mean aortic pressure is possible to demonstrate in this preparation since the heart pumps against a constant hydrostatic pressure head and the internal diameter in the aortic tubings is large This gives a low resistance to the aortic outflow The increased volume work load stimulated the glucose uptake as has been reported before (Neely *et al* 1967b) This indicates that energy metabolism can be stimulated without concomitant stimulation of the protein synthesis One objection that could be raised against this conclusion is that the very small increase in coronary flow when the work load is markedly increased could make the hearts hypoxic In favour of this hypothesis is the increased lactic acid production observed However the glycogen content was well maintained and a part of the increase in lactic acid production could be explained by the increased glucose uptake The failure of increased volume work to stimulate protein synthesis might indicate that an increased end

diastolic fiber length is not the trigger mechanism for increased protein synthetic capacity *in vitro* These results are compatible with the clinical observation that when the volume work of the heart is increased as in anemias a v shunts and in aortic regurgitation there is no consistent cardiac hypertrophy (Linzbach 1960)

The incorporation rate of phenylalanine was not linear for more than 60 min in this study thereafter a decrease in the incorporation rate was seen which has also been reported by Morgan and co workers (1971 a) They showed that the rat heart could not maintain the initial rate of protein synthesis in the retrogradely perfused heart unless the perfusion medium was fortified with five times the normal rat plasma concentrations of all amino acids An alternative explanation to the progressive decrease in protein synthesis with time is that the hearts become more dependent upon exogenous glucose as oxidative substrate since the endogenous fatty acids in the hearts are preferentially oxidized (Neely *et al* 1967 b Crass III *et al* 1969 Opie *et al* 1971) As found in this study and discussed below exogenous palmitate stimulates the protein synthesis in the perfused rat heart Other studies of cardiac protein synthesis *in vitro* with perfused preparations have indicated linear rates of incorporation for longer periods (Schreiber *et al* 1966 Kako and Minelli 1969) However the intracellular specific activities of the labelled amino acids used were not investigated in these studies The linear increase in specific activity of the cardiac proteins could in these studies be due to an increase in the specific activity of the intracellular free amino acid pool For phenylalanine it has been shown (Morgan *et al* 1971 a) that a very rapid equilibration with the intracellular free amino acid pool occurs and that the intracellular specific activity is very stable with time Moreover phenyalanine hydroxylase has been reported to be lacking in muscle (Udenfriend and Cooper 1952) and phenylalanine is not converted to other amino acids in the heart muscle (Morgan *et al* 1971 a) This finding makes phenylalanine especially convenient for studying heart protein synthesis It could be questioned whether the decline in protein synthesis during perfusion makes it difficult to registrate true stimulatory effects What is considered to be an increase in the rate of protein synthesis could be attributed to a less rapid decrease in the efficiency of the protein synthetic machinery It must however be remembered that *in vitro* conditions are not optimal for the cardiac cellular metabolism Factors as absence of insulin and thyroxine inadequate substrate supply and denervation are most likely of importance for optimal rates of protein synthesis

The finding of Rannels *et al* (1970) that palmitate stimulated the protein synthesis in the Langedorff heart compared to when glucose was used was confirmed in this study with the working heart preparation This is a strong argument for adequate oxygen supply to the perfused working heart since it has been reported (Challoner and Steinberg 1966) that the oxygen requirement is increased when palmitate is used as substrate The stimulatory effect of increased amino acid concentrations earlier reported (Morgan *et al* 1971 a) was also confirmed The observation that increased pressure load could further increase the protein synthesis in presence of palmitate and elevated amino acids levels indicates that even higher

rates of incorporation could be maintained The fact that increased afterload also can stimulate protein synthesis when substrate and amino acid supply are optimal for the incorporation of amino acids strengthens the hypothesis that the increase in protein synthesis associated with increased pressure work is of physiological significance

No consistent correlation was found between incorporation rates of phenylalanine and the distribution ratio of the same amino acid The distribution ratio was decreased in some experiments when the protein synthesis was stimulated This may indicate that a later step in the protein synthesis was accelerated more than the influx of phenylalanine However in a following paper (Ahren *et al* 1972) it is shown that increased afterload also stimulates the membrane transport of amino acids when the uptake is measured by the non utilizable amino acids *a* aminoisobutyric acid (AIB) and cycloleucine

The degree of stretch or tension seems to be important for the protein metabolism as indicated by several studies in skeletal muscles Goldberg (1967) has shown that after tenotomy of the rat gastrocnemicus muscle there is an immediate rapid and striking hypertrophy of the synergistic soleus and plantaris muscle This compensatory hypertrophy occurs also in hypophysectomized rats which excludes requirement of hypophyseal hormones for this process It has also been shown (Sciaffano and Hanzlikova 1969) that this hypertrophy can be induced in denervated muscles which suggests that non neural stretch factors are operating during compensatory hypertrophy in skeletal muscles In addition stretch applied to skeletal muscles *vitro* has been shown to increase the incorporation rate of leucine ^{14}C (Buresova *al* 1969)

An increased preload did not stimulate the incorporation of labelled amino acid in this study although according to the principles of La Place s Law there is an increase in wall stress by increased chamber volume The most striking mechanical difference between the hearts with an elevated preload and those with an increased afterload was the markedly increased end systolic residual volume in the latter ones The persistant increase in fiber length during both diastole and systole could explain the difference between pressure load and volume load However the increased end systolic volume was a result of the constriction of the aortic tube with a pronounced increase in aortic pressures and it is not certain that the increase in end systolic volume was a prerequisite for the stimulation of protein synthesis When the afterload is increased the heart must contain elevated wall forces resulting from the associated elevated systolic pressure that is generated by the ventricle and it is suggested that an increase in mural force of contraction leads to an increase in protein synthesis

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In vitro Studies on the Uptake of Biogenic Amines by Rat Mast Cells

By

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Abstract

HEISLER S and B UVNÄS *In vitro studies on the uptake of biogenic amines by rat mast cells* Acta physiol scand 1972 *86* 145—154

Mast cells from rat peritoneal and thoracic cavities were isolated and incubated with biogenic amines (dopamine [DA] noradrenaline [NA] and 5-hydroxytryptamine [5 HT]) or their precursor amino acids Competition for uptake saturation of mast cell amine stores (for 5 HT and DA) and blockade of uptake by metabolic inhibitors indicate that simple passive diffusion does not suffice to explain the uptake by mast cells of the amines studied The uptake of 5 HT and DA appears to primarily involve active transport whereas the uptake of Hi and NA may preferentially operate by facilitated diffusion

Furano and Green (1964) showed that isolated rat mast cells are capable of taking up and storing histamine (Hi) and 5 hydroxytryptamine (5 HT) According to these authors dopamine (DA) and noradrenaline (NA) are not taken up however in the present experiments mast cells were observed to be capable of also taking up these two amines

The present work attempted to study the mechanism of the transmembrane movement of the above biogenic amines by determining some of the factors which affect their uptake To this end the effect of variations in extracellular amine concentration time enzyme inhibitors and inter amine competition were investigated

Methods

Isolation of mast cells

The method of Thon and Uvnäs (1966) was used for obtaining and isolating mast cells from the peritoneal and pleural cavities of male Sprague Dawley rats (350—400 g b w) After isolation by density gradient centrifugation in Ficoll the cells were pooled An aliquot was stained with toluidine blue and the cells counted in a Burker chamber

Incubation and washing of cells

Pooled cells were suspended in a buffered (pH 6 9) salt solution containing 131 mM NaCl 8 4 mM KCl 0 81 mM $CaCl_2$ 4 3 mM Na_2HPO_4 2 3 mM KH_2PO_4 and 1 mg/ml human

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serum albumin One ml aliquots of suspended cells (1 5 to 3 5 × 10 cells) were added to plastic centrifuge tubes Immediately prior to incubation 10 μl of labelled amine were added to the tubes Unless otherwise stated, radioactive amines were used in the following concentration/ml incubation fluid 5 HT C^{14} 20 nmol DA C^{14} 10 nmol NA H^3 1 5 nmol Hi C^{14} 20 nmol Non labelled substances were added prior to incubation in volumes of 10 μl where possible Cells were incubated in a water bath (with agitation) at 37 C Details concerning the duration of incubation are given in RESULTS

Following incubation the cells were centrifuged at 350 × *g* for 10 min the supernatants were decanted and set aside The precipitated cells were washed 3 times—to remove extracellular label—in the following manner the inside of each test tube while still in an inverted position was wiped with Kleenex tissue in order to prevent backflow of the suspension medium The cells were then resuspended in 2 ml of the buffered salt solution and again centrifuged at 350 × *g* for 10 min the supernatants were decanted and kept The above procedure was repeated twice To minimize the spontaneous release of intracellular label all centrifugations and washing procedures were carried out at 4 C

Cell lysis

The precipitated cells obtained after the third wash were disrupted in one of 2 ways a) Cells were suspended in 1 ml of 0 9 % NaCl in 1 mM HCl and heated for 5 min at 65—70 C in a water bath Cells disrupted in this manner were centrifuged at 350 × *g* for 10 min the supernatants were decanted and an aliquot used for measuring radioactivity and in some experiments for paper chromatography b) Cells were suspended in 1 ml of buffered salt solution used in reincubation experiments (see RESULTS) and centrifuged at 2700 × *g* for 30 min The 2700 × *g* supernatants were decanted precipitates were resuspended in 1 ml of 1 mM HCl heated for 5 min at 65—70 and centrifuged for 10 min The supernatants were decanted and represent the 2700 × *g* precipitate Aliquots of both 2700 × *g* phases were used for measuring radioactivity and endogenous histamine

Re extraction of the precipitates (obtained from both disruption procedures) for residual label was found to be unnecessary

Analysis

a) *Measurement of radioactivity*

Radioactivity was measured using a Tri Carb liquid scintillation spectrometer Model 3375 (Packard Instrument Co) The scintillation system consisted of a mixture of 2 5 diphenyl oxazole 0 4 % w/v and of 1 4 bis 2 (4 methyl 5 phenyloxazolyl) benzene 0 01 % w/v in equal volumes of toluene and ethylene glycol monoethyl ether (Bruno and Christian 1961) To 15 ml each of the scintillation fluid were added 500 μl aliquots of the 3 washes and the supernatants obtained following cell lysis, 10 μl aliquots were added from the first supernatant obtained following incubation Radioactivity diminished successively with each washing and the amounts present in the last wash never exceeded the activity obtained from the lysed cells As a result in the latter experiments the first and second washes were pooled with the first supernatant obtained 10 μl aliquots of this mixture were also added to 15 ml of scintillation fluid

Data presented are corrected for background and efficiency

b) *Measurement of histamine*

Histamine was measured by the spectrophotofluorometric method of Shore Burkhalter and Cohn (1959) Prior extraction of histamine with butanol and heptane was not necessary since Bergendorff (personal communication) found that there are no substances present in mast cell extracts which interfere with the fluorescence of histamine Histamine values are expressed as the free base

c) *Paper chromatography*

Lysed cells were centrifuged and 50 μl of supernatant (in 10 μl aliquots) were applied to the centre of a 4 cm wide strip of Whatman no 1 paper Ascending paper chromatography was carried out overnight (~ 18 h) in a solvent system consisting of a mixture of isopropanol ammonium hydroxide (sp gr 0 88) water 20 1 2 One cm lengths of each dried strip were cut into smaller pieces and placed in counting vials containing 15 ml of scintillation fluid Radioactivity was measured by the procedure described above Reference chromatograms were spotted with unlabelled authentic standards and run concurrently After drying these were stained with 0 5 % w/v ninhydrin in acetone

Materials

The following isotopes were obtained from New England Nuclear Corp Boston Mass USA DL-noradrenaline 7 H^3 (N) 6 6 Ci/mmol 5 hydroxytryptamine ? C^{14} binoxalate 2 8 Ci/mmol and histamine H^3 (G) dihydrochloride 5 Ci/mmol The radiochemicals listed below were obtained from The Radiochemical Centre Amersham England 3 4-dihydroxyphenyl (ethyla mine ? C^{14}) hydrochloride 40 mCi/mmol and 55 mCi/mmol and histamine (ring ? C^{14}) di hydrochloride 54 mCi/mmol

Ficoll was purchased from AB Pharmacia Uppsala Sweden Amine precursors amines and enzyme inhibitors were obtained from regular commercial sources and where applicable their concentrations are expressed in terms of the free bases All other reagents used were *pro analysi* and were also obtained from commercial sources

Results

Uptake of radioactive biogenic amines as a function of extracellular amine concentration

The uptake of exogenous labelled amines was measured in mast cells following 90 min incubation In some experiments fixed amounts of radioactive amine (see Methods for concentrations) were diluted before addition to the incubation medium To estimate the maximal uptake of each amine a fixed quantity of the labelled amine was incubated with increasing amounts of unlabelled amine the results obtained were subsequently corrected for isotope dilution

As may be seen from the uptake curves (Fig 1) 5 HT and DA behave differently from NA and Hi The uptake of the former amines is greater and reaches maximum levels at extracellular amine concentrations below 100 *mμmol*/ml The uptake of NA and Hi is very much smaller at these extracellular amine concentrations and does not rise steeply until the extracellular amine concentration exceeds 500 *mμmol*/ml (This may be indicative of a preferential mode of uptake of 5 HT and DA

In order to be sure that radioactivity recovered in mast cells was not due to the enzyme formation of labelled metabolic products the following experiments were performed Cells were incubated for 90 min with 20 and 40 mμmol/ml of 5 HT C^{14} and then disrupted and centrifuged Chromatograms of both supernatants contained only one radioactive spot the Rfs of which (0 74) were identical to the Rf of authentic 5 HT Cabut and Haegermark (1966) also observed only one spot following chromatography of lysed mast cell which had previously been incubated with Hi H^3

Time course of amine uptake

Mast cells were incubated with 5 HT C^{14} (20 *mμmol*/ml) DA CA^{14} (10 *mμmol*/ml) and NA H^3 (1 5 *mμmol*/ml) for a duration of time varying between 5 and 120 min Fig 2 shows the uptake of all 3 amines increases as a function of incubation time Uptake of Hi H^3 in the experiments of Cabut and Haegermark (1966) was directly proportional to the duration of incubation from 5 to 320 min

Competition between amines for uptake by mast cells

Mast cells were incubated for 90 min in the presence of a fixed amount of one radioactive amine and increasing concentrations of the other unlabelled amines in order to investigate the competition between amines for uptake

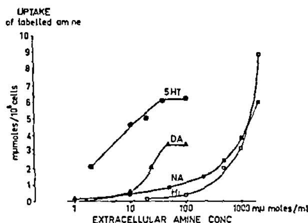


Fig 1

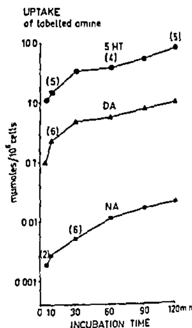


Fig 2

Fig 1 Uptake of radioactive biogenic amines by rat mast cells as a function of extracellular amine concentration Cells were incubated for 90 min at 37 C Each point represents the mean of 2 (5 HT and DA) or 3 (NA and Hi) expts

Fig 2 Uptake of radioactive biogenic amines by rat mast cells as a function of the duration of incubation Cells were incubated at 37 C Each point represents the mean of 3 expts except where indicated by parentheses ●——● 5 HT (20 mμmol/ml ■——■ (1 5 mμmol/ml) ▲——▲ DA (10 mμmol/ml)

a) *The effect of DA NA and Hi on uptake of 5 HT C^{14}*

Fig 3 a indicates that NA (HCl) Hi (diHCl) and DA (HCl) did not compete with 5 HT C^{14} for uptake even in concentrations up to 100 mμmol/ml *i e* 5 times the 5 HT concentration DA showed an inhibitory effect although high concentrations (600 mμmol/ml) were necessary for this effect to become manifest

b) *The effect of 5 HT NA and Hi on uptake of DA C^{14}*

The data obtained in this experiment are summarized in Fig 3b 5 HT (oxalate) was an effective inhibitor of DA C^{14} uptake in both equi and subequimolar concentrations An extracellular 5 HT concentration of 4 mμmol/ml produced a 50 % inhibition By contrast Hi and NA only produced inhibition in very high concentrations

c) *The effect of 5 HT DA and Hi on uptake of NA H^3*

Uptake of NA H^3 (Fig 3 c) was inhibited by 5 HT DA and Hi In inhibiting 50 % of the uptake 5 HT was 10 times more effective than DA and 120 times more effective than Hi

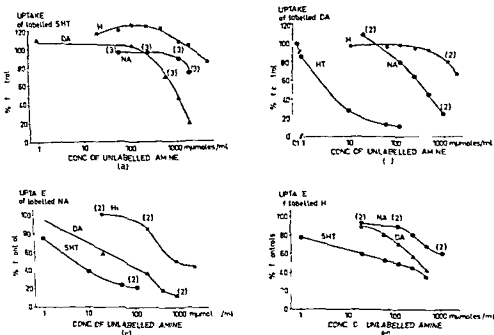


Fig 3 Competitive uptake of biogenic amines by rat mast cells Cells were incubated for 90 min at 37 C Radioactive amines were used in the following concentrations/ml incubation fluid 5-hydroxytryptamine C^{14} (5-HT) 20 mμmol dopamine-C^{14} (DA) mμmol nor adrenaline H^3 (NA) 1 5 mμmol histamine C^{14} (Hi) ?0 mμmol The effects of unlabelled biogen c amines are represented as follows 5 HT ●——● DA ▲——▲ NA ○——○ Hi ■——■ a) the effect of DA NA and Hi on the uptake of 5-HT C^{14} Each point represents the mean of 2 expts except where denoted by parentheses b) the effect of 5-HT NA and Hi on the uptake of DA C^{14} Each point represents the mean of 4 expts except where denoted by parentheses c) the effect of 5-HT DA and Hi on th uptake of NA H^3 Each point represents th mean of 3 expts except where denoted by parentheses d) the effect of 5 HT DA and Na on the uptake of Hi C^{14} Each point represents the mean of 4 expts except where denoted by parentheses

TABLE I The effect of 5-HT and DA on endogenous histamine content of rat mast cells In all experiments cells were concomitantly incubated with 20 n mol/ml extracellular Hi Incubation time 90 min at 37 C Data represent the mean of 2 expts

Amine added	Concentration (nmol/ml)	Hi released n total cellular content	μg Hi/10⁶ cells (free base)
Control	—	12 6	29 7±1 8
5 HT	20	12 0	31 2±1 ?
	120	11 6	32 1±1 2
	480	9 5	31 3±1 0
DA	10	14 4	31 0±2 0
	1?0	11 8	32 4±0 1
	480	7 6	31 9±0 0

TABLE II The effect of unlabelled amines on the content of labelled amines taken up by preincubation of rat mast cells. Cells were preincubated for 60 min (37 °C) with 5 HT C^{14} (20 nmol/ml), DA C^{14} (10 nmol/ml) or Hi C^{14} (20 nmol/ml), washed 3 times and reincubated for 15 min (37 °C) in the presence of 200 nmol/ml of unlabelled amine. The figures given represent the mean of 3 expts.

Radioactive amine	Added amine	Radioactivity released as % of total activity taken up
5 HT C^{14}	Control	3.8 ± 0.3
	DA	8.3 ± 0.2
	NA	7.3 ± 1.4
	H	3.9 ± 0.2
DA C^{14}	Control	12.9 ± 1.0
	5 HT	13.1 ± 0.9
	NA	14.6 ± 0.1
	H	14.7 ± 0.6
Hi C^{14}	Control	18.2 ± 1.3
	5 HT	21.6 ± 3.4
	DA	17.5 ± 1.7
	NA	19.0 ± 1.6

d) *The effect of 5 HT, DA and NA on uptake of Hi C^{14}*

In order of potency, 5 HT was more effective an inhibitor of Hi C^{14} uptake than DA or NA (Fig. 3 d). Only 5 HT exerted an inhibitory effect on Hi C^{14} uptake when used in subequimolar concentrations. Uptake was inhibited 50 % when the extracellular concentration of 5 HT was 5.5 times greater than the concentration of Hi and when the concentration of DA was 16 times greater.

In summary, NA and Hi were only moderately effective in blocking the uptake of each other; they were both poor inhibitors of 5 HT C^{14} and DA C^{14} uptake. On the other hand, 5 HT and DA were effective in blocking the uptake of NA H^3 and Hi C^{14}. 5 HT was a very effective inhibitor of DA C^{14} uptake and DA was also capable, though less effectively so, of blocking 5 HT C^{14} uptake.

e) *Competition between amines for cellular storage*

The inhibition of the uptake of a labelled amine into mast cells by another unlabelled amine may be due not only to competition for transport; there might also be an apparent inhibition due to competition for storage sites and consequent release of the endogenous amine. A reduced uptake of label could then also be interpreted on the basis of substitution of unlabelled for labelled amine at the binding site. Such a phenomenon has been demonstrated in murine mastocytoma cells (Mannaioni, Fischer and Garman 1968) in which 5 HT causes release of Hi and *vice versa*. In order to test for a similar event in the present experiments, mast cells were incubated for 90 min with unlabelled Hi and increasing concentrations of 5 HT and DA. NA was not tested since it has been shown previously to have no effect on Hi content of rat mast cells in an extracellular concentration of 450

TABLE III The effect of amine precursors on uptake of radioactive amines by isolated rat mast cells incubated for 90 min at 37° C

Radioactive amine	Uptake of radioactive amine in percentage of control (mean of 3 expts)											
	Amine Precursor (nmol/ml)											
	L Histidine				L DOPA				DL-5 HTP			
	20	250	500	1 000	20	250	500	1 000	20	250	500	1 000
5 HT C	98.1 ±0.3	98.0 ±1.6	96.7 ±1.7	90.7 ±3.4	88.3 ±2.5	78.1 ±0.8	73.0 ±1.0	71.0 ±1.0	100.0 ±7.0	86.8 ±2.9	90.3 ±1.1	87.6 ±2.6
DA C	98.7 ±1.8	95.5 ±1.4	94.5 ±2.1	89.8 ±1.8	101.4 ±1.7	96.8 ±0.5	102.3 ±4.2	107.3 ±3.0	89.4 ±2.8	86.9 ±1.0	83.0 ±1.9	81.4 ±5.9
NA H	—	90.3 ±9.2	96.4 ±1.9	94.7 ±1.7	—	83.9 ±3.4	80.5 ±7.8	73.9 ±1.6	—	92.5 ±6.0	92.0 ±5.8	93.5 ±2.6

mμmol/ml (Frisk—Holmberg personal communication) Table I indicates that 5 HT and DA were incapable of reducing Hi content A second more expansive set of experiments was also performed in which cells preincubated with label for 60 min were washed 3 times and reincubated for 15 min with 200 mμmol of unlabelled amine Table II illustrates that the cold amines produced no detectable major effect on the content of isotopic amines taken up by the cells during preincubation It is therefore reasonable to assume that the competition between amines for uptake in the present exprnments does not involve intracellular competition between amines for storage sites

The effect of amine precursors on amine uptake

To determine if amine precursors also inhibited the uptake of preformed biogenic amines labelled amines were incubated for 90 min with increasing amounts of unlabelled L histidine L dihydroxyphenylalanine (L DOPA) and 5 hydroxy DL-tryptophan (DL-5 HTP) The data obtained are presented in Table III The uptake of each of the labelled amines was not appreciably affected by concomitant incubation with any of the amine precursors (The uptake of Hi H^3 is similarly not affected by the presence of DL-5 HTP or L histidine (Cabut and Haegermark 1966)

The effect of enzyme inhibitors on amine uptake

Isolated cells were incubated at 37° C for a 60 min period in the presence of labelled amine and varying concentrations of well known enzyme inhibitors The results obtained are summarized in Table IV The most potent inhibitory effect in this series of experiments was exerted by N-ethylmaleimide followed by ouabain and KCN In order of response to the inhibitors the uptake of 5 HT C^{14} was most affected followed by DA C^{14} NA H^3 and Hi C^{14} Ouabain and KCN did not markedly affect the uptake of NA H^3 and Hi C^{14}

TABLE IV The effect of enzyme inhibitors on uptake of radioactive amines by isolated rat mast cells incubated for 60 min at 37 C Inhibitors were added immediately prior to the addition of the radioactive label

Radioactive amine	Uptake of radioactive amine in presence of inhibitor as a percentage of uptake in controls (mean of 3 expts)								
	Enzyme inhibitor (nmol/ml)								
	Ouabain			N Ethylmaleimide			KCN		
	10	100	1000	10	100	1000	10	100	1000
5 HT C14	92 2 ±2 5	79 9 ±1 2	38 1 ±0 6	39 4 ±0 5	29 3 ±0 6	16 5 ±0 3	100 3 ±1 4	46 8 ±0 7	3[illegible] 7 ±0 7
DA C14	98 9 ±1 0	89 9 ±1 4	62 2 ±1 9	49 7 ±0 1	37 7 ±0 2	27 2 ±1 1	97 4 ±1 1	69 0 ±0 8	39 1 ±0 7
NA H3	94 4 ±2 1	96 9 ±2 5	81 8 ±2 5	81 3 ±0 6	70 9 ±1 1	57 0 ±1 1	106 0 ±1 7	93 6 ±1 2	115 6 ±8 9
Hi C14	94 1 ±0 3	92 8 ±1 4	81 5 ±1 6	—	—	61 0 ±1 6	—	104 7 ±3 8	109 9 ±4 7

Discussion

The transport of substances across cell membranes according to current concepts may proceed by three different routes a) simple passive diffusion — substances move down a concentration or electrochemical gradient b) specialized active transport — characterized by selectivity saturability and utilization of metabolic energy (Ussing 1949, Danielli 1954) and c) facilitated diffusion — characterized by selectivity and saturability with no input of free energy being required other than that needed to maintain the structure of the cell membrane (Stein 1967)

The ability of amines to compete for uptake saturation of mast cells with increasing extracellular amine concentration (5 HT and DA), the blockade of amine uptake by metabolic inhibitors (esp 5 HT and DA) indicate that simple passive diffusion *per se* does not suffice to explain the uptake of the amines studied Since intracellular amines are stored in high concentration in granules a concentration gradient between the intracellular store and the extracellular medium exists Therefore though it would be impossible to completely eliminate diffusion as a means by which amines enter mast cells the data obtained indicate that amines must undergo active transport and/or facilitated diffusion on their way to the granule sites

The active transport of amines may be linked to the simultaneous transport of sodium ions a system which has been used to explain the uptake of sugars (Crane 1965) amino acids (Kipnis and Parrish 1965) and NA (Bogdanski and Brodie 1969) by various tissues Active transport of sodium ions in turn requires metabolic energy believed to be provided by an ATP hydrolyzing enzyme — the so-called (Na K) ATPase (Caldwell 1956 1960 Whittam 1958 Caldwell *et al* 1960 Hoffman 1960 Skou 1963) A relationship between ionic and amine movement in mast cells is indirectly supported by the following observations active transport of sodium ions is characteristically inhibited by cardiac glycosides (Glynn 1957 Whittam 1958) Inhibition of amine uptake by ouabain has been previously reported in different tissues

(Hughes Shore and Brodie 1958 Dengler Spiegal and Titus 1961 Bertie and Shore 1967 Bogdanski and Brodie 1969) In the present experiments ouabain inhibited the uptake in varying degrees of all the amines Furthermore N-ethyl maleimide which is an inhibitor of ATPase (Skou 1963) also blocked the uptake of the amines tested Incubation of mast cells in sodium free medium (sucrose or lithium substitution) also inhibited the uptake process (unpublished data) A similar sodium requirement was demonstrated for NA uptake in sympathetic nerve endings (Bogdanski and Brodie 1966) and in isolated perfused rat hearts (Horst Kopin and Ramey 1968)

The f ct that metabolic inhibitors did not produce a 100 % blockade of uptake of any amine and that KCN was completely ineffective in this respect to Hi and Na suggests that active transport may not be the only process responsible for the movement of amines into mast cells but that uptake of the amines may also proceed independently of energy production Since passive diffusion is probably of minor significance transport of this nature may be due to facilitated diffusion

Evaluation of the factors affecting the uptake process suggests that the uptake of 5 HT and DA is different in many respects from the uptake of Hi and NA Mast cells for instance were saturated with 5 HT and DA in much lower extracellular concentrations than were required of Hi and NA The uptake of labelled 5 HT and DA was less affected by the presence of unlabelled amines than was the uptake of labelled Hi and NA Metabolic inhibitors were more effective in blocking the uptake of 5 HT and DA than Hi and NA

The separation of the amines into two groups on the basis of their reactivity to a number of different factors raises the possibility that the uptake of amines by mast cells may be due to preferential utilization of one mode of transport for instance active transport over another ie facilitated diffusion Based on saturability (maximal uptake at relatively lower extracellular concentrations) selectivity (competitive ability) and energy requirements (more marked effect of metabolic inhibitors) the uptake of 5 HT and DA appears to primarily involve active transport whereas the uptake of Hi and NA may preferentially operate by facilitated diffusion Such categorization of course requires further substantive investigation

Aside from the mode of uptake the data obtained raise a question relative to the function of the uptake process itself Mast cells can decarboxylate histidine (Schayer 1956) 5 HTP (Lagunoff and Benditt 1959) and DOPA (Slorach and Uvnas 1968) Furthermore the amine precursors (5 HTP DOPA histidine) do not block the uptake of labelled amines indicating that preformed amines can enter mast cells independently of the uptake and possible decarboxylation of their respective precursors Consequently since amines in mast cells may be sequestered there regardless of the ability of the cell to synthesize such amines the uptake process itself may accordingly serve to remove biogenic amines from the circulation under either normal or pathogenic conditions The mast cell thus acting as a repository for amines may play an active role in the homeostatic regulation of the levels of circulatory amines The ability to inactivate amines through uptake has been described for the uptake of 5 HT by platelets (Markwardt 1968)

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The Effect of Bilateral Carotid Artery Ligation upon the Blood Flow and the Energy State of the Rat Brain

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Abstract

Eklof B and B K Siesjo *The effect of bilateral carotid artery ligation upon the blood flow and the energy state of the rat brain* Acta physiol scand 1972 86 155—165

The cerebral effects of bilateral carotid artery ligation were studied in lightly anesthetized rats either alone or combined with moderate reductions in the mean arterial blood pressure The relative decreases in cerebral blood flow were estimated from the a v differences for O_2 and CO_2 on the assumption of an unchanged cerebral metabolic rate Ligation of the carotid arteries alone was found to decrease cerebral blood flow to about 50 % of normal but to give no significant changes in the energy state of the tissue as judged from the tissue contents of the ATP ADP and AMP When the cerebral blood flow was reduced further by means of an induced decrease in blood pressure the energy state of the tissue was severely affected At this point the cerebral venous P_{O_2} was reduced from about 50 to about 30 mm Hg Since pure hypoxemia with a reduction of venous P_{O_2} to about 10 mm Hg can be tolerated without changes in the energy state of the tissue it is concluded that ischemia may lead to a grossly inhomogenous reduction in the tissue perfusion

Although it is well known that a progressive reduction of the cerebral circulation ultimately leads to disturbances of the oxidative metabolism of the brain it is not known how far the flow can fall before the oxygen supply becomes insufficient This is partly due to the fact that the cerebral circulation shows a pronounced autoregulation (Lassen 1959 Rapela and Green 1964 Harper 1965 Haggendal and Johansson 1965 Zwetnow 1970) and that when the cerebral perfusion pressure has been reduced to values below the autoregulatory range there is a precipitous fall in flow The narrow pressure range within which the flow changes from a normal to a nutritionally inadequate flow corresponds to a marked threshold for the occurrence of biochemical changes (Siesjo and Zwetnow 1970 a and b Siesjö *et al* 1971) hampering the correlation between an intermediate fall in flow and its metabolic consequences Thus present knowledge on the minimal flow compatible with an essentially unchanged oxidative metabolism stems from experiments with pronounced hyperventilation which may decrease cerebral blood flow to 50 % of

the normal with only moderate metabolic changes (Alexander *et al* 1968 Granholm *et al* 1969 Miller *et al* 1970 Granholm and Siesjo 1971) However the altered tissue CO tension may not be entirely without effects on the O requirement of the tissue and it cannot be concluded from these experiments that the tissue can withstand a 50 % reduction in flow also in normocapnic or hypercapnic situations.

The relation between cerebral blood flow and cerebral metabolism may be more easily studied by ligating one or several of the arteries supplying the brain but recent experiments have shown that at least in the dog and in the monkey three of the main supplying arteries can be ligated without significant effects on the cerebral blood flow as measured with electromagnetic flow meters or with radioactive xenon (Eklof and Schwartz 1969 1970) In the present experiments, we therefore combined bilateral carotid artery ligations in the rat with moderate reduction in the mean arterial blood pressure by bleeding the animals The results have shown that the cerebral blood flow as estimated from the a v O and CO differences, is markedly lowered by the ligation and that it can be further reduced if the mean arterial blood pressure is lowered to values within the autoregulation range (100 and 70 mm Hg respectively) In the present communication the reduction in cerebral blood flow has been correlated to the cerebral venous P_O and to the cerebral energy state as judged from the tissue concentrations of phosphocreatine ATP ADP AMP and inorganic phosphate A following communication (Eklof and Siesjo 1972) will describe the corresponding changes in the intra and extracellular lactate and pyruvate concentrations in the intracellular pH and in the tissue contents of carbohydrate substrates A preliminary account of the present findings has published (Eklof and Siesjo 1971)

Methods

Most of the experimental procedures and techniques used in the present experiments have been described in detail in preceding communications from the laboratory (Siesjo and Zwetnow 1970 a Nilsson and Siesjo 1970 Siesjo and Nilsson 1971) For that reason only the general outline of these procedures and techniques will be described and emphasis will be laid only on new techniques

Operative techniques Male Wistar rats (300—400 g) were anesthetized with divinyl ether tracheotomized immobilized with tubocurarine chloride and connected to Starling type respirators which delivered a gas mixture with 70 % N_2O and 30 % O_2 at a rate that gave arterial CO_2 tensions of 35—40 mm Hg One femoral artery was cannulated for blood pressure recording with an electromanometer and for anaerobic sampling of blood The superior sagittal sinus was exposed for anaerobic sampling of cerebral venous blood (Pontén and Siesjo 1966 Brzezinski *et al* 1967) and the atlantooccipital membrane was exposed for sampling of cisternal cerebrospinal fluid (CSF) A plastic funnel was fitted into a skin incision over the skull bone for subsequent freezing of the brain *in situ* (see Siesjo and Nilsson 1971) With this freezing technique the supratentorial part of the brain are frozen in about 30 s but since an adequate arterial oxygenation is upheld during the whole freezing period and since the circulation of deeper tissue regions seems to be upheld until these regions are reached by the freezing front autolytic changes appear to be avoided The body temperature which was measured in the rectum was kept close to 37 C

In order to allow carotid artery ligation the common carotid arteries were dissected free in the neck and loops of ligature were placed around the vessels The ends of the ligatures were allowed to project through the sutured wounds and the ligatures could later be closed by pulling their ends

Analytical techniques Arterial and cerebral venous blood was analysed at 37 C for pH P_{CO_2} and P_{O} using microelectrodes and arterial blood was also analysed for the hemoglobin lactate and pyruvate contents The CSF samples were used for analyses of either the total CO_2 content (Siesjo 1962) the CO_2 tension (Ponten and Siesjo 1966) or the lactate and pyruvate concentrations The brain samples which consisted of the whole supratentorial parts of one hemisphere of the brain were stored at −80 C and subsequently analysed for phosphocreatine ATP ADP AMP inorganic phosphate (P_i) lactate pyruvate α keto-glutarate glucose and glutamate For all metabolites except P_i (see Wahler and Wollenberger 1958) specific enzymatic techniques were used after extraction of the tissue at −15 C (Lowry *et al* 1964 see also Siesjo and Nilsson 1971)

The decrease in cerebral blood flow due to the carotid artery ligation was calculated on the assumption of an unchanged cerebral metabolic rate for O_2 and CO_2 Thus provided this assumption is valid the relation between the blood flow in the experimental situation (E) and in the control situation (C) will be given by the ratio $(AVD)_C/(AVD)_E$ In the groups in which the carotid arteries were ligated without lowering of the blood pressure the a v differences were derived for both CO_2 and O_2 The a v CO_2 differences were measured with a microdiffusion method (see Siesjo 1962) in four control experiments and related to the simultaneously measured P_{CO} differences Since the relation gave a straight line relationship the a v CO_2 differences were derived from the P_{CO} differences using this line (see below)

In the groups in which the mean arterial blood pressure was lowered there was an increase in the tissue lactate content (see below) Since this lactacidosis might give rise to a nonsteady state with regard to the CO_2 production in the tissue the relative decrease in CBF was calculated from the a v O_2 differences The arterial and the venous O_2 contents were calculated from the P_O the pH and the hemoglobin content using an O_2 dissociation curve with a P_{50} of 38 mm Hg (Hall 1966 see also Handbook of Respiratory Data) assuming that the pH correction for human blood (see Kelman and Nunn 1966) is valid also for rat blood

Experimental protocol In connection with the other operative procedures loose ligatures were placed around the common carotid arteries The animals were turned positioned in the headholder and allowed a 20—30 min steady state period The animals were then divided into 2 main groups In the first main group arterial and cerebral venous P_O P_{CO} and pH were measured at times zero 5 and 30 min Immediately after the 30 min samples cisternal CSF was sampled for the measurement of the CO_2 tension In this main group the animals were divided into four subgroups In the first of these which served as a control group no carotid artery ligation was performed and arterial and venous blood as well as CSF were sampled at 30 min In the second subgroup the carotid arteries were ligated and the blood pressure was allowed to attain its spontaneous value In the third and fourth subgroups the ligations was done when the mean arterial blood pressure had been lowered to 100 and 70 mm Hg respectively In groups 2—4 arterial and venous samples were taken before the ligation (0 min) and 5 and 30 min respectively after the ligation

In the second main group no venous measurements were performed CSF was sampled for measurements of lactate and pyruvate or for the total CO_2 contents and the tissue was frozen for measurements of metabolites This main group was divided into the same types of subgroups as described above In the control group CSF was sampled and the brain was frozen after 30 min In groups 2—4 these procedures were performed after 5 and 30 min respectively The two main series of experiments were intermingled to ensure that the operative and experimental procedures were similar In both series arterial blood was sampled repeatedly for measurements of pH P_{CO} P_O and in the end of the experiment for hemoglobin content lactate and pyruvate

Results

Blood parameters The mean hemoglobin concentrations in the groups varied between 15 1 and 15 8 g/100 ml and there was no significant lowering of the hemoglobin content in any of the groups which were bled to give mean arterial blood pressures of 100 and 70 mm Hg respectively The body temperature was close to 37° C in all groups (36 8—37 2°) The blood gas data for arterial and cerebral venous blood have been given in Table I together with the mean arterial blood pressures The arterial P_{O_2} values were well above 100 mm Hg in all groups The carotid

TABLE I The effects of carotid artery ligation in normotensive and hypotensive rats on mean arterial blood pressure (mm Hg) blood gases (mm Hg) and pH in arterial and cerebral venous blood Means ± S E Figures within parenthesis indicate the number of experiments Statistical differences between the ligated groups and the control group as calculated with students T test were indicated as follows *p < 0 05 **p < 0 01 ***p < 0 001

Experimental group	MABP	Arterial Blood			Sup sag sinus		
		P_{O_2}	P_{CO_2}	pH	P_{O}	P_{CO_2}	pH
Controls	165±2 (44)	120±3 (44)	37 5±0 4 (44)	7 38±0 01 (43)	51 5±1 3 (35)	48 8±0 9 (27)	7 32±0 01 (35)
Carot lig (5 min)	180±6 (28)	115±4 (?8)	37 2±0 7 (28)	7 35±0 01 (28)	41 4±1 5*** (20)	55 4±1 1*** (17)	7 28±0 01 (20)
+hypotension	100 (19)	115±5 (17)	33 7±1 0*** (17)	7 27±0 0? (19)	32 1±1 6*** (11)	56 3±? 8*** (7)	7 22±0 03 (13)
	70 (19)	129±5 (19)	31 5±1 5*** (19)	7 20±0 02 (19)	28 6±1 7*** (13)	60 5±2 5*** (8)	7 06±0 03 (13)
Carot lig (30 min)	160±5 (25)	1?3±4 (25)	38 7±0 9 (25)	7 28±0 01 (25)	36 2±1 2*** (19)	62 8±1 2*** (17)	7 17±0 0? (19)
+hypotension	100 (25)	130±4 (25)	33 2±1 1*** (25)	7 20±0 03 (25)	32 9±1 7*** (16)	60 5±2 7*** (12)	7 06±0 03 (16)
+hypotension	70 (20)	133±5 (19)	30 ?±1 7*** (19)	7 12±0 02 (19)	27 8±2 2*** (14)	65 8±2 6*** (11)	7 01±0 03 (14)

artery ligation did not by itself affect the arterial P_{CO_2} but the lowering of the mean arterial blood pressure was found to be associated with the same type of fall in ... al P_{CO} as has previously been found to occur during arterial hypotension in the rat (Kaasik *et al* 1970 Siesjo and Zwetnow 1970 a) The arterial pH decreased after carotid ligation presumably due to a lactacidosis elicited by peripheral vasoconstriction and this decrease was greatly exaggerated if the blood pressure was lowered

Ligation of the carotid arteries gave rise to a significant fall in the venous P_{O_2} which was further pronounced if the mean arterial blood pressure was lowered to 100 or to 70 mm Hg The increased a v P_O difference was accompanied by a corresponding widening of the P_{CO_2} difference from 11 3 mm Hg in the control group to 30—35 mm Hg in the 70 mm Hg groups

Cerebral blood flow As remarked in the introduction the influence of the carotid artery ligation upon the cerebral blood flow was estimated from the a v CO and O differences on the assumption of an unchanged cerebral metabolic rate Since this assumption is probably only valid for the normotensive groups (see below), the values given for the hypotensive groups will only indicate the minimum decrease in flow encountered In the normotensive groups the relative decrease in flow was calculated both from the CO and the O a v differences Fig 1 shows that there was a linear relationship between the a v differences for total CO and for P_{CO_2} in a series of control experiments without carotid artery ligation The straight line

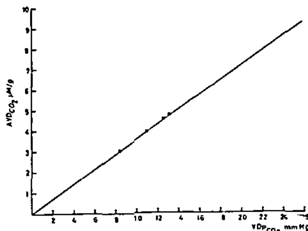


Fig 1 The relation between a v differences (femoral artery superior sagittal sinus) for P_{CO} and simultaneously measured total CO_2 contents (determined by a microdiffusion method) in 4 control expts The line was extrapolated from the best visual fit to the points

drawn as the best fit to the points was used to derive the a v differences for total CO in the groups The decreases in flow calculated from the CO differences are shown in Table II together with those calculated from the a v O differences The figures indicate that carotid artery ligation alone decreased CBF to 60 % of the normal value at 5 min and to 50 % at 30 min the validity of the calculation being *trengthened by the correspondence between the values obtained from the a v dif* ferences for CO and O respectively The values further indicate that when the blood pressure was lowered to 100 mm Hg or lower there was a decrease in CBF to 45 % of normal or less 30 min after the carotid artery ligation

In Fig 2 the cerebral venous O tension was related to the cerebral blood flow as calculated from the a v O differences in controls and in rats with carotid ligation for 5 and 30 min respectively A reduction in flow to 50 % of the normal in the 30 min ligation group was as ociated with a lowering of the venous P_{O_2} from 51 mm Hg to 36 mm Hg The lowest Pv_O s encountered (25—30 mm Hg) cor responded to reductions in flow to 40 % of normal or lower

TABLE II Th effects of carotid artery ligation n normotens ve and hypotensi e rats on CBF in % of controls estimated from the arter ovenous differences of CO and O Means ± S E There were between 10 and 18 expts in each group

Exp group	CBF in % of control	
	AVD$_{CO}$	AVD$_{O_2}$
Carot d ligation 5 m n		
> 160 mm Hg	63±3	56±3
100 mm Hg	48±2	46±2
70 mm Hg	39±2	41±1
Carot d ligat on 30 min		
> 160 mm Hg	50±2	50±1
100 mm Hg	*48±3*	*44±1*
70 mm Hg	34±2	42±1

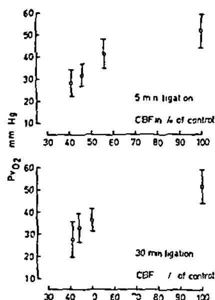


Fig 2 The relation between the cerebral blood flow as calculated from the a v differences for O and the venous O_2 tension in the superior sagittal sinus in (from right to left) controls and in rats with carotid artery ligation at MABP of > 160 100 and 70 mm Hg respectively The upper graph gives values at 5 min of ligation and the lower graph after 30 min of ligation Means ± S D

Brain tissue changes

Table III gives the measured tissue parameters in the control group as well as in the carotid artery ligated groups The control values for the phosphates agree closely with values previously reported from the laboratory (Nilsson and Siesjo 1970 Siesjo and Zwetnow 1970 b Siesjo and Nilsson 1971) Ligation of the carotid arteries alone did not lead to significant changes in ATP ADP AMP or P_i but in the 30

TABLE III The effects of carotid artery ligation in normotensive and hypotensive rats on the phosphocreatine ATP ADP AMP and inorganic phosphate concentrations in brain tissue (mMol/kg wet tissue) Figures within parenthesis indicate the number of experiments Means ± S E For explanation of statistical signs see Table I

Experimental group	PCr	ATP	ADP	AMP	P_i
Controls	4.89	2.85	0.42	0.04	1.98
(6)	±0.05	±0.04	±0.03	±0.007	±0.18
Carotid lig 5 min					
> 160 mm Hg	4.87	2.75	0.47	0.04	2.19
(6)	±0.10	±0.07	±0.03	±0.003	±0.30
100 mm Hg	3.33*	2.61	0.54	0.34	3.74
(6)	±0.61	±0.26	±0.11	±0.18	±1.04
70 mm Hg	1.34 **	2.11*	0.83***	0.44*	6.25 *
(6)	±0.30	±0.25	±0.07	±0.14	±1.12
Carotid lig 30 min					
> 160 mm Hg	4.59	2.73	0.33	0.04	2.10
(6)	±0.09	±0.12	±0.07	±0.004	±0.26
100 mm Hg	2.92	2.08	0.65	0.41	7.79
(8)	±0.66	±0.34	±0.11	±0.18	±1.92
70 mm Hg	0.64*	0.68 *	0.59	1.23***	12.81 *
(5)	±0.48	±0.35	±0.10	±0.26	±2.25

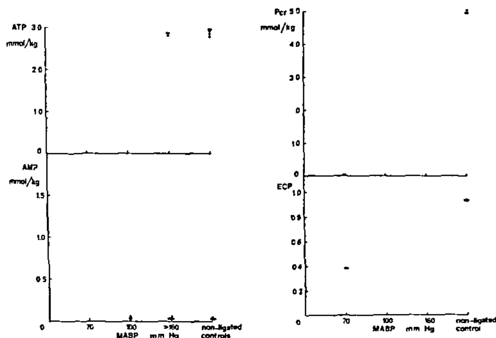


Fig 3 The effects of carotid artery ligation for 30 min upon the ATP AMP and phosphocreatine concentrations and upon the energy charge potential in rat brain tissue The figure compares the values obtained in an unligated control group to those obtained after bilateral carotid artery ligation at three different blood pressure levels

min group there was a significant fall in phosphocreatine ($p < 0.05$) However since the fall in phosphocreatine may be attributed to the decrease in intracellular pH through an effect on the creatine phosphokinase equilibrium (see Siesjo and Messeter 1971 Messeter and Siesjo 1970) we may conclude that a reduction in CBF to about 50 % of normal (Table II) was unassociated with a significant effect on the energy state of the tissue In order to obtain a quantitative expression for the energy state the energy charge potential of the adenine nucleotide pool

$$\frac{(ATP) + 0.5\ (ADP)}{(ATP) + (ADP) + (AMP)}$$

(Atkinson 1968) was calculated for the groups The calculation corroborated the conclusion that bilateral carotid artery ligation alone did not affect the energy state of the tissue ($p > 0.05$)

When the mean arterial blood pressure was maintained at 100 mm Hg and the carotid arteries were ligated the mean values for phosphocreatine and ATP fell and there were increases in ADP AMP and P However with the exception of phosphocreatine these changes were not statistically significant At a mean arterial blood pressure of 70 mm Hg carotid artery ligation lead to highly significant changes in PCr ATP AMP and P_i ($p < 0.01$)

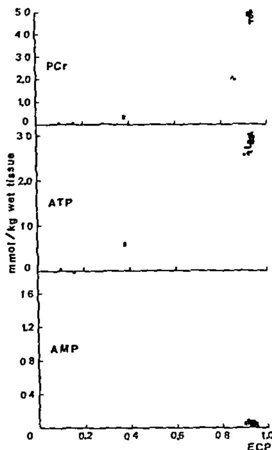


Fig 4 The relation between the energy charge potential of the cerebral adenine nucleotide pool and the brain tissue concentrations of phosphocreatine ATP and AMP (in mMol/kg wet tissue) in unligated control animals as well as in all the animals with bilateral carotid ligation

In Fig 3 the individual values for ATP AMP and phosphocreatine and for the energy charge potential (ECP) have been given for the 30 min groups At a mean blood pressure of 100 mm Hg four out of eight animals showed marked decreases in phosphocreatine and ATP and in the energy charge potential as well as marked increases in AMP The findings suggest that the decrease in cerebral blood flow induced by ligating the carotid arteries at a mean blood pressure of 100 mm Hg is marginally critical for the energy metabolism of the brain Thus the further reduction in flow encountered with a reduction in blood pressure to 70 mm Hg invariably and drastically changed all parameters shown in Fig 3

There was a good linear correlation between on one hand the decrease in the energy charge potential and on the other hand the corresponding changes in ATP and AMP (Fig 4) However the relation between phosphocreatine and energy charge potential suggests that phosphocreatine may fall in the absence of a change in the energy charge potential This fall seems to be related to a decrease in the intracellular pH occurring in all ligated animals even in the absence of changes in the energy state (see Eklof and Siesjo 1972)

Discussion

The present results have demonstrated that bilateral carotid artery ligation in lightly anesthetized rats is accompanied by changes in the a-v O and CO differences suggestive of a 50 % reduction of the blood flow in the supratentorial parts of the brain. The results have further demonstrated that this reduction in flow does not lead to a significant change in the energy state of the tissue. The results are thus completely in accordance with those which have shown that although vigorous hyperventilation reduces cerebral blood flow by about 50 % the oxidative metabolism and the energy state of the tissue remain essentially unchanged (see Introduction).

The method of calculating cerebral blood flow from the a-v O and CO differences is probably adequate in normoxia and during moderate cerebral hypoxia but since severe hypoxia is apt to decrease the O consumption of the brain the method can only indicate the minimal decrease in flow in situations of pronounced tissue hypoxia. We may conclude from the present data that a reduction of the cerebral blood flow to below 45 % of normal is just sufficient to alter the energy state of the tissue. Thus pronounced hyperventilation seems to decrease cerebral blood flow to values which are close to the critical values for the energy state of the tissue.

It has recently been shown that hypoxemia with a reduction of the arterial P_O to 15—20 mm Hg is unaccompanied by significant changes in the energy charge potential of the brain provided the blood pressure of the animals does not fall (MacMillan and Siesjö 1971). Since these P_{vO} values are associated with a cerebral venous P_O of 10 mm Hg or less a capillary tissue P_{O_2} gradient of less than 10 m Hg appears sufficient to maintain oxidative phosphorylation. In the present experiments a marked fall in the energy charge potential occurred at a venous P_O of about 30 mm Hg implying that the venous P_{O_2} measured under the present ischemic conditions poorly reflects the end capillary P_O, values. The discrepancy between the results is probably due to grossly inhomogenous reduction of flow in the ischemic brains. Thus the derangement of the energy state of the tissue may be explained by a drastic fall in flow to parts of the tissue some of which may even be unperfused and the relatively high venous P_{O_2} measured in ischemic brains could then primarily reflect end capillary P_O in well perfused tissue regions. The inhomogenous reduction in flow could either occur at the macroscopic level with relatively large areas being deprived of a blood supply or it could occur as a patchy capillary perfusion. However whatever is the true explanation the present experiments suggest that the cerebral venous P_O measured under ischemic conditions cannot readily be interpreted in terms of critical P_{O_2} gradients between capillary blood and tissue. The results further suggest that the decrease in flow at low perfusion pressures may be grossly inhomogenous and that a given reduction in global flow may be associated with regional flow differences perhaps ranging from nonperfusion to a relatively unchanged flow.

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Effect of Training on Esterified Fatty Acids and Carnitine in Muscle and on Lipolysis in Adipose Tissue in Vitro

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Abstract

FROBERG S O I ÖSTMAN and N O SJOSTRAND *Effect of training on esterified fatty acids and carnitine in muscle and on lipolysis in adipose tissue in vitro* Acta physiol scand 1972 *86* 166—174

The concentration of triglycerides phospholipids and carnitine was determined in red and white parts of the gastrocnemius muscle from rats with and without a period of training by running Also the rate of lipolysis in adipose tissue was determined with and without stimulation *in vitro* The concentration of triglycerides was reduced in the red and white muscle tissue but positively correlated to each other in white muscle tissue This suggests that the white muscle tissue Correlation analysis revealed no direct relationship between the spontaneous or stimulated rate of lipolysis *in vitro* and the triglyceride concentration in either type of muscle tissue This suggests that the level of muscle triglycerides was influenced or determined by other factors than the rate of mobilization of fatty acids from the adipose tissue per se The concentrations of carnitine and triglycerides were negatively correlated in red muscle tissue but positively correlated to each other in white muscle tissue This suggests that the metabolic role of carnitine with regard to triglyceride metabolism differed between the two muscle types The possibility was discussed that the capacity of the intracellular carnitine transport system for fatty acids into mitochondria of red muscle tissue increased in the course of physical training as part of an adaptation towards enhanced oxidative metabolism

Studies on the metabolism of plasma free fatty acids (FFA) during exercise have suggested that the endogenous muscle triglycerides besides the plasma FFA contribute with fatty acids to the oxidative metabolism (Havel *et al* 1963 Havel *et al* 1964, Havel *et al* 1967 Havel *et al* 1967 Issekutz *et al* 1964 Issekutz and Paul 1968) This hypothesis is substantiated by the finding of a decrease in skeletal muscle triglyceride concentration after exercise in both man (Froberg and Mossfeldt 1971 Carlson *et al* 1971 and rat (Froberg 1971) The rapid decrease in the muscle triglyceride concentration when the mobilization of plasma FFA from the adipose tissue was inhibited with nicotinic acid (Carlson *et al* 1966) also indicates that the endogenous muscle triglycerides are a metabolically active lipid fraction which is integrated into the metabolism of the plasma FFA

TABLE I Body weight and weight of hearts and adrenals in untrained and trained rats

	Body weight g		Heart weight g	Adrenal weight mg
	Initial	Final		
Untrained (N=13)	324±4²	459±3	1 32±0 01	42±2
Trained (N=11)	316±4	42?±2	1 41±0 01	5?±2
p		< 0 001	< 0 001	< 0 001

¹ Number of rats
² Mean±standard error of the mean
indicates degree of statistical significance for difference between group means of untrained and trained rats

Little is however known about the effect of training on the fatty acid metabolism at rest and during exercise There are evidence that the utilization of plasma FFA is increased during exercise in well trained man (Havel *et al* 1963 Havel *et al* 1964) and dog (Paul and Issekutz 1967 Issekutz *et al* 1965) In the rat physical conditioning reduced the concentration of muscle triglycerides (Carlson and Fröberg 1969 Froberg 1971) as well as of plasma FFA (Froberg 1971) Furthermore we recently observed that the resting muscle triglyceride concentration was negatively related to work performance in man and that during exercise the decrease in the muscle triglyceride concentration was inversely related to the amount of fatty acids oxidized (Carlson *et al* 1971) A lowered muscle triglyceride concentration after training may thus indicate that physical conditioning influenced the metabolism of fatty acid during exercise by increasing the fractional contribution of plasma FFA to the energy metabolism during exercise Since one determinant for the availability of plasma FFA is the rate of mobilization from the adipose tissue we have compared the muscle triglyceride concentration with the rate of mobilization from the adipo e tissue *in vitro* of untrained and trained rats Results are also presented on the effect of training on the muscle concentration of carnitine which has been reported to be of major importance for the oxidation of long chain fatty acids by transferring the FA into the mitochondria (Fritz 1967)

Material and methods

Male Sprague Dawly rats were trained for 15 weeks by daily runs on a moto driven treadm ll The tra ning intensity was gradually increased and d ring the last 5 weeks of the training period the rats were able to run for 80 min at a speed of 37 m/min Details pertaining to the training p ogram have been published el ewhere Östman and Sjostrand 1971) Bod weight, heart weight and weight of adrenals in trained and untra ned rats are gi en n Table I The increased weight of heart and adrenals in the trained ats ndicates that the training was efficient

Sampling was done on nonfa ting ats bet een 9 a m and noon 4 days after the last run The rats were decapitated after light ether anesthesia Both epididymal fat pads were removed and each d id d in four equal pieces perp nd cular to the long axis About 100 mg from the

TABLE II Concentration of desoxyribonucleic acid (DNA) and phospholipids in epididymal adipose tissue from untrained and trained rats

	DNA mg/g	Phospholipids mg/g
Untrained (N=10)[1]	0 29±0 02[2]	0 80±0 03
Trained (N=10)	0 48±0 03	1 03±0 05
p[3]	< 0 001	< 0 001

[1] Number of rats
[2] Mean ± standard error of the mean
[3] Indicates degree of statistical significance for difference between group means of untrained and trained rats

corresponding regions of the fat pads were then put into one vial *e g* each rat gave adipose tissue to 4 vials one for each of 4 incubation groups The vials were arranged so that each incubation group contained adipose tissue from the four regions of the fat pad Incubation was done in Krebs Ringer bicarbonate buffer containing 3 % of albumin (Kabi Stockholm Sweden) and 0 1 % of glucose The rate of lipolysis was estimated as the release of glycerol (Wieland 1957) to the medium between 10 and 70 min of incubation at 37 C Noradrenaline (Norexadrine® AB Astra Sodertalje Sweden) and caffein (Sigma Chemical Co Sigmachem St Louis USA) were used to stimulate lipolysis saline was added to the control vials

When the incubation was ended the adipose tissue from the same rat was pooled frozen in liquid nitrogen and stored at −15 C The desoxyribonucleic acid (DNA) concentration was determined according to Schneider (1957) The phospholipids were extracted with chloroform methanol (Carlsson 1963) The chloroformphase was passed through a silic acid column then the column was washed with chloroform and the phospholipids eluted with methanol as previously described (Micheli *et al* 1969) for determination of their concentration (Carlson 1960)

Both gastrocnemic muscles were removed not later than 3 min after the animals were killed immediately frozen in liquid nitrogen and stored at −15 C After thawing the muscle tissue was separated into macroscopically red and white tissue and analysed for triglycerides and phospholipids as described earlier in detail (Froberg 1967) with the modification that the muscle tissue was freezedryed before lipid analysis The carnitine concentration including acetylcarnitine and free carnitine in red and white muscle tissue was determined according to Cederblad[1] and Lindstedt (1972)

Results

Adipose tissue

The concentration of DNA in the adipose tissue was increased from 0 29 mg/g in the untrained rats to 0 48 mg/g in the trained rats and that of phospholipids from 0 80 to 1 03 mg/g (Table II)

The spontaneous as well as the stimulated rate of glycerol release expressed per g wet weight was increased in the trained rats (Table III) With noradrenaline stimulation the mean rate of glycerol release was 2 26 and 3 41 μmol per g and hour in control and trained rats respectively Caffeine alone or together with noradrenaline gave the same lipolytic response as noradrenaline alone

[1] We are indepted to Dr Cederblad Dept of Clinic Chemistry Sahlgrenska sjukhuset Gothenburg Sweden who carried out the carnitine analysis

TABLE III Rate of lipolysis in vitro in epididymal adipose tissue from untrained and trained rats. The rate of lipolysis was estimated as the glycerol release to the medium from 10 to 70 minutes of incubation at 37 C
Additions to the medium Noradrenaline to a concentration of 1 µg/ml Caffeine to a concentration of 2 12 mg/ml Saline was added to the control vials

Addition to the medium	Glycerol release µmol/g/h			
Noradrenaline µg/ml	0	1 0	0	1
Caffeine mg/ml	0	0	2 12	2 12
Untrained (N=10)¹	0 81±0 03²	2.26±0 14	2 39±0 05	2 66±0 06
Trained (N=10)	1 0±0 05	3 41±0 23	3 25±0 22	3 84±0.24
P	< 0 01	< 0 001	< 0 01	< 0 001
	Glycerol release µmol/mg DNA/h			
Noradrenaline µg/ml	0	1 0	0	1
Caffeine mg/ml	0	0	2 12	2 12
Untrained (N=10)	2 95±0 27	8 04±0 70	8 89±0 83	9 87±0 87
Trained (N=10)	2 14±0 18	7 23±0 56	6 90±0 51	8 20±0 68
P	< 0 05	> 0 05	> 0 05	> 0 05

¹ Number of rats
² Mean ± standard error of the mean
³ Indicates degree of statistical significance for difference between group means of untrained and trained rats

When the rate of lipolysis was related to the DNA concentration instead of the wet weight the spontaneous rate of lipolysis was reduced from 2 95 to 2 14 µmol per g DNA and hour ($p < 0 05$) in the trained rats while the stimulated glycerol release did not differ between trained and untrained rats (Table III)

The individual differences between the spontaneous and the noradrenaline stimulated lipolysis were 1 45±0 15 and 2 41±0 20 µmol per g wet weight and hour (M+S E) in control and trained rats respectively Related to the DNA concentration the corresponding values became 5 09±0 58 and 5 19±0 45 µmol per mg DNA and hour When however the individual difference between spontaneous and stimulated lipolysis per mg DNA and hour was calculated as per cent change setting the spontaneous glycerol release to 100 the mean value for trained rats 345± 21 % tended to be higher than for untrained rats 282 ± 22 % ($p < 0 05$)

Skeletal muscle

In the red muscle tissue the concentration of triglycerides decreased and the concentration of phospholipids increased The carnitine concentration was elevated in

TABLE IV Concentration of triglycerides phospholipids and carnitine in red and white muscle of untrained and trained rats

	Triglycerides μmol/g d w		Phospholipids mg/g d w		Carnitine mg/g d w	
	R	W	R	W	R	W
Untrained	8.09 ± 0.38[2]	9.80 ± 0.99	43.8 ± 1.0	36.8 ± 0.9	0.66 ± 0.02	0.64 ± 0.02
N	13	13	11	12	13	13
Trained	4.74 ± 0.39	6.22 ± 0.99	46.8 ± 0.8	37.0 ± 1.1	0.77 ± 0.03	0.62 ± 0.02
N	11	11	10	11	8	8
p	< 0.001	< 0.05	< 0.05	> 0.05	< 0.001	> 0.05

R = red and W = white muscle tissue
[1] Number of rats
[2] Mean ± standard error of the mean
[3] Indicates degree of statistical significance for difference between group means of untrained and trained rats

the red muscle from 0.66 ± 0.02 in untrained rats to 0.77 ± 0.03 mg per g dry weight in trained rats (Table IV) In the white muscle tissue the triglyceride concentration decreased while no change was observed in the concentration of phospholipids and carnitine

In Table V linear correlation coefficients are listed for some of the variables investigated The noradrenaline stimulated glycerol release calculated per g wet weight was negatively correlated to the concentration of triglycerides in the red and white muscle tissue while no correlation was found between the triglyceride concentration in the two muscle types and the spontaneous glycerol release When the rate of lipolysis was expressed on the basis of the DNA concentration in the adipose tissue the correlation between the stimulated glycerol release and the muscle triglyceride concentration disappeared

The body weight was negatively correlated to the DNA concentration in the adipose tissue This suggests that the lowered body weight in trained rats was due to loss of adipose tissue triglycerides resulting in an increased cellularity per unit wet weight adipose tissue *This is also in accordance with the positive correlation between* the concentration of DNA and phospholipids in the adipose tissue

The body weight was correlated both to the stimulated glycerol release and the muscle triglyceride concentration When the influence of body weight on these variables was eliminated by *partial regression analysis no independent* correlation was found between the noradrenaline stimulated glycerol release and the triglyceride concentration in the two muscle types

The carnitine concentration was negatively correlated to the triglyceride concentration in red muscle tissue and positively correlated to the triglyceride concentration in the white muscle tissue The carnitine concentration was positively correlated to the phospholipid concentration in red as well as in white muscle tissue

TABLE V Correlation coefficients for some of the variables investigated In the correlation analysis values for untrained and trained rats have been combined

x	y	N	r	p
L	TG_R	20	−0.60	< 0.01
L	TG_W	20	−0.55	< 0.05
L	TG_R	20	−0.42	> 0.05
L	TG_W	20	−0.18	> 0.05
L_{DNA}	TG_R	20	0.19	> 0.05
L_{DNA}	TG_W	20	0.10	> 0.05
BW	DNA_{AT}	20	−0.68	< 0.001
BW	L	20	−0.72	< 0.001
BW	TG_R	20	−0.81	< 0.001
BW	TG_W	20	−0.52	< 0.05
P_{AT}	DNA_{AT}	20	0.62	< 0.01
C_R	TG_R	21	−0.72	< 0.001
C_W	TG_W	21	0.53	< 0.05
C_R	P_R	18	0.56	< 0.05
C_W	P_W	20	0.50	< 0.05
Partial regression anlysis				
L TG_R	BW	20	−0.04	> 0.05
L TG_W	BW	20	−0.10	> 0.05

N *number of observations*
r correlation coefficient
p indicates the degree of statistical significance for r values
C_R carnitine concentration in red and C_W in white muscle tissue
L spontaneous and L noradrenaline stimulated lipolysis per gram wet weight and L_{DNA} per mg DNA in the adipose tissue
TG_R triglyceride concentration in red and TG_W in white muscle tissue
P_R phospholipid concentration in red and P_W in white muscle tissue
P_{AT} concentration of phospholipids and DNA_{AT} concentration of DNA in adipose tissue
BW indicates body weight

Discussion

Noradrenaline is believed to enhance the lipolysis by increasing the level of cyclic AMP caffeine by inhibiting the breakdown of cyclic AMP (Butcher and Sutherland 1965 Butcher 1970) In the present study the lipolytic activity was stimulated with 1 μg noradrenaline per ml medium which has been reported to cause maximal stimulation of lipolysis (Wenke 1970) With caffeine alone the glycerol release was increased to about the same extent as with noradrenaline and together noradrenaline and caffeine did not further enhance the rate of the glycerol release suggesting that the activity of the lipase enzyme system in the adipose tissue was maximally stimulated Thus although physical conditioning increased the stimulated lipolysis per unit wet weight no effect was observed per unit DNA concentration *e g* per unit cell Similar results have been reported on the adrenaline stimulated lipolysis from adipose tissue *in vitro* from trained and untrained rats (Parizkova *et al* 1966)

Muscle triglycerides accumulate labelled palmitic acid *in vitro* (Masoro *et al* 1966 Beatty and Bocek 1970) and *in vivo* (Jones and Havel 1967) There are evidence that the level of muscle triglycerides *in vivo* is intimately related to the

availability of plasma FFA (Carlson *et al* 1966) and at rest the plasma FFA concentration was reduced in trained compared to untrained rats as was also the muscle triglyceride concentration (Froberg 1971) In the present study the spontaneous rate of glycerol release on the basis of the DNA concentration was reduced in adipose tissue from trained compared to untrained rats If this also reflects a lowered rate of mobilisation of plasma FFA *in vivo* the availability of plasma FFA for esterification to triglycerides in muscle tissue may have decreased and thus also the muscle triglyceride concentration It should be emphasized however that relating the rate of lipolysis to the DNA concentration may be misleading since other cells than adipocytes may have contributed to the DNA concentration of the adipose tissue and furthermore loss of adipose tissue interspersed between muscle fibres may have contributed to the lowered muscle triglyceride concentration in the trained rats

The plasma FFA (Havel *et al* 1964, Havel *et al* 1967 Havel *et al* 1967, Issekutz *et al* 1964, Issekutz and Paul 1968) as well as the muscle triglycerides contribute with fatty acids to the oxidative metabolism during exercise (Froberg and Mossfeldt 1971 Carlson *et al* 1971 Froberg 1971) The lowered muscle triglyceride concentration in the trained rats suggests reduced availability of fatty acids from endogenous stores of triglycerides Physical conditioning may thus have adapted the fat metabolism during exercise towards increased utilization of plasma FFA This is to some extent supported by exercise studies in man (Havel *et al* 1963 Havel *et al* 1964) and dog (Paul and Issekutz 1967 Issekutz *et al* 1965) of different degree of physical fitness The correlation analysis did however not indicate that the muscle triglyceride concentration was determined by the rate of lipolysis in vitro This however permits no conclusion with regard to a relationship between the metabolism of plasma FFA and the metabolism of muscle triglycerides under in vivo conditions since 1) the total amount of adipose tissue was not known and 2) the rate of lipolysis differ in adipose tissue from different sites in the body (Carlson and Hallberg 1968, Carlson *et al* 1969) Furthermore the flow of plasma FFA to the muscle in vivo and thus the uptake into the muscle is dependent not only by the rate of mobilisation of plasma FFA from adipose tissue but also by the blood flow to the muscle A change in the muscle hemodynamics in the course of training may therefore have influenced on the local turnover of plasma FFA in the muscles

Carnitine has been reported to increase the rate of oxidation of longchain fatty acids (*cf* Fritz 1967) and to decrease the rate of incorporation of fatty acids into triglycerides in rat heart slices in vitro (Fritz 1964) The elevated concentration of carnitine in the red muscle tissue may indicate that the training increased the transport capacity of fatty acids into mitochondria in vivo and thus shunted fatty acids into metabolic pathway leading to the fatty acid oxidase enzyme system rather than into glyceride synthesis The negative correlation between the concentration of triglycerides and carnitine in the red muscle tissue lends some support for this hypothesis Furthermore training has been reported to have adaptive effects on muscle mitochondria both by increasing their number (Gollnick and King 1969) and their capacity for oxidative metabolism (Holloszy 1967)

It is of interest that the response of training on the carnitine concentration differed between red and white muscle tissue Differences between red and white muscle tissue have however been observed with regard to lipid composition (Fröberg 1967) capillary density (Romanul 1965) blood flow (Reis and Wooten 1970) enzymatic equipment and metabolism (Keul Doll and Keppler 1969) The present findings may indicate that also the role of carnitine in the intracellular metabolism of fatty acids differ between red and white muscle tissue

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Regulation of Middle Ear Sound Transmission in the Nonanesthetized Rabbit

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Abstract

BORG, E. *Regulation of middle ear sound transmission in the nonanesthetized rabbit.* Acta physiol. scand. 1972. 86. 175—190.

The regulation of sound transmission through the middle ear of nonanesthetized rabbits was quantitatively investigated by comparing the excitation of the cochlea before and after surgical inactivation of m. stapedius and/or m. tensor tympani in the stimulated ear. The crossed middle ear reflex was used as a measure of the excitation of the cochlea to stimulation with pure tones in the range from 0.5 to 8.0 kHz at intensities up to 125 dB SPL. Sounds through this frequency range were attenuated above a level of 90 to 100 dB SPL. Low frequency tones (0.5 kHz) were attenuated by all levels of activity in the m. stapedius and m. tensor tympani. High frequencies were attenuated only by m. stapedius activity at high intensity levels above reflex threshold (20—30 dB). The m. tensor tympani had no influence on high frequencies above 2.0 kHz. Improved sound transmission due to reflex activity was not regularly found. The efficiency of the regulation was regarded as high: a 1.0 dB increase in sound level in the ear canal gave only a 0.3 dB increase in the excitation of the cochlea (*i.e.* the regulating efficiency of the closed loop system was 0.7 dB attenuation per 1.0 dB increase in stimulus sound intensity). The regulating efficiency of the open loop system was about 2 dB/dB. The open loop gain was estimated to 4 during steady state. The oscillations in the middle ear reflex response were found to be generated in the m. stapedius feed back loop regulating the sound transmission through the middle ear. It was concluded that the function of the acoustic middle ear reflexes can be described as extending the dynamic range of the cochlea and adaptation and it may also be compared with the effect of lateral inhibition.

Contractions of m. stapedius and m. tensor tympani have usually been found to attenuate the sound transmitted through the middle ear in experiments on narcotized animals (Wiggers 1937, Wever and Vernon 1955, Møller 1965, Price 1966). In these acute experiments the cochlear microphonic potentials were used to measure the output of the middle ear and the middle ear muscles were stimulated with contralateral sound (Wever and Vernon 1955, Møller 1965, Price 1966), electrically (Møller 1965) or pharmacologically (Wiggers 1937, Gisselsson *et al.* 1957, Gerhardt and Wagner 1966). The recordings were made with the wall of the middle ear opened. Openings in the wall of the middle ear (or the acoustic bulla) have however recently been shown to change the acoustic properties of the middle ear (Møller 1965, Guinan and Peake 1967, Tonndorf and Khanna 1967) and also to change the effect of the middle ear reflexes (Borg 1972 a).

The regulation of the sound transmission has also been studied with chronically implanted electrodes at the round window of cats (Galambos and Rupert 1959 Simmons 1963 Carmel and Starr 1963) and in the brain stem (Marsh and Worden 1969) However these studies give very incomplete information on the dependence on the frequency and intensity of the sound A quantitative investigation of the regulation of sound transmission through the middle ear throughout a wide intensity and frequency range under physiological conditions is thus needed

The aim of the present study was to determine the influence of m stapedius and m tensor tympani separate and combined (*i e* the total reflex) on the transmission of sound of various frequencies and intensities through the middle ear in non anesthetized unrestrained rabbits Some aspects of the temporal pattern of the reflex were also studied The origin of the oscillations in the reflex response amplitude discussed in relation to regulation of sound transmission *e g* by Lorente de Nó (1935) Wersall (1958) and Møller (1962) were analysed experimentally

The crossed middle ear reflex was used in measuring the level of excitation in the cochlea (Borg 1972 b) Comparisons of the crossed stimulus response curves obtained before and after surgical inactivation of m stapedius and/or m tensor tympani in the stimulus ear gave a quantitative measure of the regulation provided by the two muscles

Methods

The method for sound stimulation and for recording the middle ear muscle activity as changes in the acoustic impedance (at 800 Hz) was comprehensively described by Borg (1972 c) as well as operative procedures and specifications of the material Some additional information pertinent to the present study will be added

Use of the middle ear reflex in measuring changes in transmission properties of the middle ear When one or both middle ear muscles in the stimulated ear are inactivated the energy transmitted to the cochlea is changed compared to the situation with both muscles active This change was measured quantitatively by recording the reflex response in the opposite ear to acoustic stimulation before and after inactivation of the muscles The crossed reflex was thus used as a means of measuring the level of excitation in the cochlea of the stimulated ear The shift of the stimulus-response curve (the distance along the abscissa) gave quantitative information on the influence of the muscles on the sound transmission (see Fig 2 and also Borg 1968 The ipsilateral reflex in the normal ear was used as a reference and the response values were expressed as percentage of the maximal obtainable response amplitude in the nonoperated ear to ipsilateral stimulation It was also used to check the stability of the recording conditions

The reliability of the shift of the stimulus response curve as a measure of the regulation of sound transmission is dependent on the degree of variability (reproducibility) normally present in the reflex responses at repeated measurements The standard deviation (SD) of the shift parallel to the intensity axis was found to be less than 2 0 dB and the SD for the change in slope was 1 0 dB see Borg 1972 b) In the present study usually at least two curves before and three curves after the operations were averaged before the shift was determined This procedure increases the stability of the determination of the change in sound transmission (Borg 1972 b)

Results

Dynamic properties

Oscillations have frequently been observed in the closed loop response of the middle ear reflex (see *e g* Borg 1972 c) They are seen both ipsilaterally and contralaterally

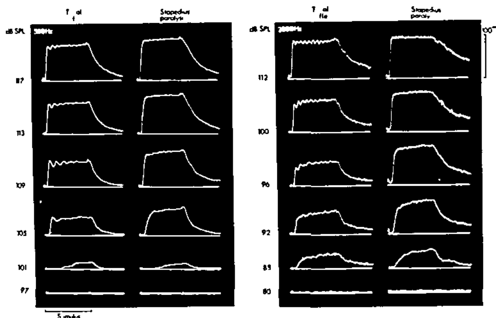


Fig 1 Time course of the response of the crossed total middle ear reflex of two nonanesthetized rabbits recorded as changes of the acoustic impedance at 800 Hz. Stimulation was 0 5 kHz (left graph two columns) and 2 0 kHz (right graph two columns) pure tone of 0 5 s duration of various intensities (legend numbers) The left columns in each graph show the responses to stimulation in the contralateral normal ear with m stapedius intact The two right columns show the responses obtained to stimulation of the contralateral ear after m stapedius had been denervated in the stimulated ear The maximal amplitude of the ipsilateral reflex response is in each case 100 % Threshold of m tensor tympani reflex in stimulus ear is 107 and 93 dB SPL for 0 5 and 2 0 kHz respectively

and are most prominent for stimulation with high intensity sound (giving 80 % to 90 % of maximal response) in the middle and low frequency range Since the cause of the oscillations has been much debated their relation to regulation of sound transmission was analysed in the present investigation A description of the open loop dynamics will also be presented here as a basis for further quantitative analysis

Origin of the oscillations Oscillations under certain circumstances are characteristic in responses of negative feed back systems Such oscillations disappear when the generating feed back loop is opened *i e* when inhibiting influence of the output signal on the input signal is abolished If the oscillations in middle ear muscle responses are generated in feed back loops in the central nervous system or as a consequence of autogenic inhibition from stretch receptors in the muscle or tendons the temporal characteristics of the crossed response will be unchanged after paralyzing the middle ear muscles in the stimulus ear If on the other hand the oscillations are generated in the feed back loop regulating the sound transmission through the middle ear they will disappear both in the crossed and the uncrossed response when the middle ear muscles in the stimulated ear are paralyzed

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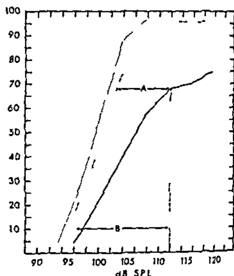


Fig 2 Stimulus response relation of the crossed total reflex to stimulation with 0 5 kHz pure tone of 0 5 s duration Continuous line muscles in stimulated ear intact Broken line m stapedius paralyzed in stimulated ear Dotted line m tensor tympani also cut in stimulated ear (open loop conditions) 100 % is in each case the maximal response in the recording ear with ipsilateral stimulation A is attenuation provided by m stapedius at B dB above its threshold (dot i e 10 % of maximal impedance change of the total reflex) before inactivation

Fig 1 shows the effect of denervation of m stapedius in the stimulated ear on the temporal characteristics of the contralateral response (where both m stapedius and m tensor tympani are intact) in two rabbits In both rabbits the denervation was performed by extracting the 7th cranial nerve through the stylomastoid foramen After paralyzing the stapedius muscle in the stimulated ear no oscillations were seen in the crossed response either at 0 5 kHz or at 2 0 kHz

Also transection of the 7th cranial nerve in the brainstem changed the temporal characteristics as shown in Fig 1 Since the oscillations disappeared in both the ipsilateral and in the (normal) contralateral ear after paralyzing the m stapedius in the ipsilateral ear the oscillations most likely originated in the m stapedius feed back loop associated with regulation of sound transmission through the middle ear

The open loop response When the contractions of the middle ear muscles do not influence the transmission of the stimulus sound the feed back loop is said to be open The open loop system is simpler than the intact system and its properties are basic for the description and understanding of dynamic properties of the feed back regulation In six animals both the m stapedius and the m tensor tympani were denervated or cut in one ear Therefore the crossed reflex responses show the dynamic properties of the open loop system Since the m tensor tympani has a neglible effect on the sound transmission above about 2 0 kHz (see below), the open loop properties may be illustrated by the crossed response with stimulation at 2 0 kHz after only the m stapedius has been inactivated in the stimulus ear

The column furthest to the right of Fig 1 shows the response of the total reflex to pure tones of 2 0 kHz after denervation of the m stapedius in the stimulated ear It was characteristically seen in the responses that the rise time decreased at increased levels of stimulus intensity No oscillations were seen The differences in time

course due to the direction of the change in sound intensity (on or off) are seen in Fig 1 The speed of onset is greater than that for decay in both the open loop and closed loop responses A quantitative analysis of the open loop characteristics is in press (Borg 1972 d)

The regulation of sound transmission

The influence of middle ear muscle activity on sound transmission through the middle ear was investigated by studying changes of the contralateral reflex activity after inactivation of the muscles in the stimulated ear The crossed reflex was thus used as a measure of the level of cochlear excitation in the stimulated ear

The m tensor tympani reflex If the m stapedius reflex is intact the influence of the m tensor tympani on sound transmission is too small to be measurable with the technique used even at low sound frequencies Only when the m tensor tympani is isolated *i e* the m stapedius having been previously paralyzed its influence on sound transmission can be measured Fig 2 shows the crossed total reflex response to stimuli of 0 5 kHz in an animal whose m stapedius was denervated in the stimulus ear (broken line) The continuous line shows the normal response of the crossed reflex obtained before the muscle was inactivated The shift of the stimulus response curve was a direct measure of the attenuation provided by the muscle before inactivation It was masured as the distance parallel to the abscissa (A) with two dB intervals above (B) the ipsilateral reflex threshold before inactivation (dot)

In a subsequent operation the m tensor tympani was also cut in the stimulus ear The responses of the crossed reflex under these conditions are shown by the dotted line It can be noted that only a small increase in the crossed reflex response amplitude is present after cutting the m tensor tympani in the stimulus ear The attenuation provided by the isolated m tensor tympani was usually below 0 2 to 0 3 dB per 1 0 dB increasee in sound level The attenuation of 0 5 kHz stimuli provided by the isolated m tensor tympani reflex usually was initiated immediately above its threshold for influence on the impedance at 800 Hz No influence of the m tensor tympani on sound transmission at and above 2 0 kHz at intensities up to 120—125 dB SPL could be measured

The m stapedius reflex The contraction of the m tensor tympani attenuates sound only to a small extent Thus the change in sound transmission after paralyzing the m stapedius shows the role of the m stapedius which is a close approximation of the situation in which the two muscles work together The change in the stimulus response curve of the crossed reflex after denervation of the m stapedius in the stimulated ear in a typical experiment is shown in Fig 3 A shift of the stimulus response curves to the left is shown following m stapedius denervation This shift indicates that the level of cochlear excitation at a given sound intensity was higher after denervating the m stapedius as when the m stapedius was active The difference between the curves was measured as shown in Fig 2

Fig 4 shows representative determinations of the influence of m stapedius contractions on sound transmission at four frequencies as a function of intensity The

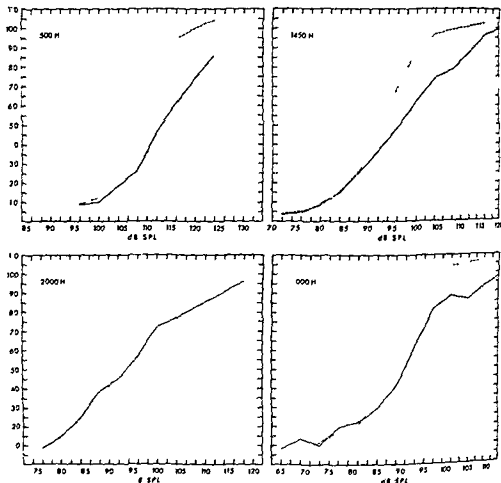


Fig 3 Stimulus-response curves of the crossed total reflex before (continuous line) and after (broken line) denervation of the m stapedius in the stimulated ear Stimulation with pure tones of 0 5 s duration (0 5 1 45 2 0 and 4 0 kHz) 100 % is maximal response of ipsilateral reflex in each case One animal

horizontal shift between the curves before and after denervation of the m stapedius in the stimulus ear (see Fig 2) was determined for successive 2 dB increments of sound intensity beginning at threshold (10 % impedance change) of the ipsilateral reflex before denervation of m stapedius The values are plotted as a function of sound intensity above the ipsilateral reflex threshold Positive values on the ordinate represent decrease in sound transmission as a result of m stapedius contraction At 0 5 and 2 0 kHz the curves are based on averages of up to six curves obtained before and after m stapedius denervation at the other frequencies only one to three curves were available Fig 4 indicates that attenuation of low frequency sound (0 5 kHz) began immediately above the ipsilateral reflex threshold whereas tones of higher frequency were attenuated only at levels well above the reflex threshold (at 4 0 kHz 25—30 dB) The significance of the small gain in transmission sometimes observed

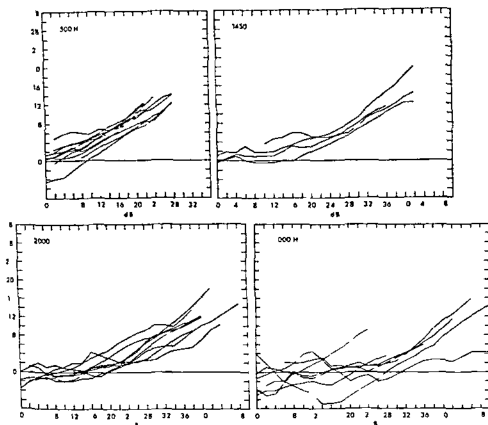


Fig 4 Attenuation of pure tones of 0 5 1 45 2 0 and 4 0 kHz as a function of sound level in dB re the ipsilateral total reflex thre hold (10 % of maximal impedance change) M staped us is denervated in the stimulated ear m. tensor tympani is intact Positive values in d cat the increase in sound pressure le el necessary to obtain the same response amplitude in the contralateral ear (measuring ear) when the m stapedius is intact as when it is paralyzed in the stimulated ear

below this level of intensity is questionable (see Fig 3) In the range where the trans mission was decreased by contraction of the m stapedius an attenuation of 0 5 to 0 8 dB per 1 0 dB increase in sound intensity was usually seen The attenuation did not saturate in the sound intensity range investigated (up to 120—125 dB SPL) despite the fact that the impedance change of the ipsilateral reflex regularly reached a plateau at a somewhat lower sound level

In natural conditions when the middle ear reflexes are activated by complex sound the relation between the response amplitude and power of various frequency components is complex The information given in Fig 4 showing attenuation as a function of intensity of a pure tone is not directly applicable Knowledge of the attenuation of different frequency components as a function of measured impedance change is easier to comprehend

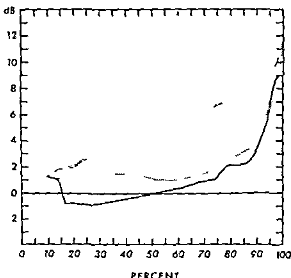


Fig 5 Attenuation at 0 5 kHz (broken line) 2 0 kHz (continuous line) and 4 0 kHz (dotted line) as a function of relative ipsilateral reflex response amplitude (per cent values on abscissa) one rabbit

Fig 5 shows the attenuation provided by the m stapedius reflex at 0 5 kHz (broken line) 2 0 kHz (continuous line) and at 4 0 kHz (dotted line) in one animal as a function of relative reflex response amplitude The high frequencies were only attenuated near maximal reflex activity (80 %—90 %) whereas the low frequency was attenuated throughout the range The difference in the attenuation between low and high frequencies was usually below 10 dB

In Fig 1 it was shown that oscillations in the reflex response only appear when the m stapedius function is normal The lowest ipsilateral response amplitude at which oscillations were observed corresponded to an attenuation of sound transmission being on an average 3 3 dB (SD = 1 7 n = 20) This relationship between time course and attenuation allowed one to determine qualitatively the effect of the middle ear muscle contraction on sound transmission from single closed loop responses At high frequencies (above 4 0 kHz) the shift of the stimulus response curve was difficult to use as a measure of the attenuation since the available sound intensity usually did not give maximal reflex responses The shape of response was instead used to study the regulation of high frequencies Fig 6 shows the reflex responses to stimuli at sound frequencies *from 2 0 kHz to 8 0 kHz* The first four recordings were from a single rabbit whereas the last recording on the right was from a different rabbit The oscillations are most clearly seen at 2 0 and 4 0 kHz but they are also present at 6 0 and 8 0 kHz The fact that oscillations are seen in these responses indicates that the middle ear reflexes attenuate sound transmission through the middle ear at least up to 8 0 kHz

The total reflex When both muscles are intact the attenuation is slightly increased at low frequencies (below 2 0 kHz) compared to the effect of the m stapedius alone There is an increase in attenuation from 0 5—0 6 dB (m stapedius alone) to 0 7—0 8 dB per 1 0 dB increase in sound level Above 2 0 kHz the sound

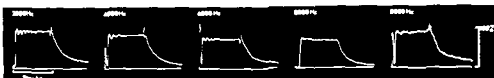


Fig 6 Time course of reflex responses to pure tone stimulation (0 5 s duration) showing various degrees of damped oscillations The four curves to the left are from one rabbit The curve to the right is from another rabbit 100 % is maximal amplitude of ipsilateral reflex response at 2 0 kHz All the responses but the second from the right were obtained at ipsilateral stimulation The stimulus levels used were 99 dB SPL 95 dB SPL 96 dB SPL 87 dB SPL and 83 dB SPL respectively from left to right

is attenuated 0 5—0 8 dB per 1 0 dB increase in sound level by the m stapedius alone and the m tensor tympani has not been found to produce any further increase in attenuation

Efficiency of regulation (Regulating efficiency)

The slope of the curves of Fig 4 expressing the relation between the attenuation (equivalent decrease in stimulus sound pressure level in dB) and the stimulus sound pressure level in dB can be defined as the efficiency of regulation of the system With this definition the efficiency of regulation of the closed loop system is usually between 0 5 and 0 8 dB/dB (see above) The use of logarithmic units is suitable since the slope is approximately constant from a few dB above the threshold value. The measure of efficiency of regulation furthermore becomes compatible with the usual way of expressing sound pressure

The open loop efficiency of regulation can also be calculated from the data of Fig 4 Regard a case with the closed loop efficiency of regulation equal to 0 7 dB/dB In the closed loop system the response to a tone 10 dB above threshold will be the same as a sound 3 dB above threshold produces in the open loop system Since this very amplitude of response corresponds to an attenuation of 7 0 dB of the closed loop system it will also do so for the open loop system It then immediately follows that the relation between attenuation and stimulus sound level (re reflex threshold) will have a slope of 7 0/3 0 = 2 3 dB/dB In words A sound stimulus the level of which is 10 dB above the open loop reflex threshold will elicit a middle ear muscle contraction that is capable of attenuation of the sound equivalent to a 23 dB decrease in sound pressure level In the same way the open loop efficiency of regulation can be estimated to 1 0 to 4 0 dB/dB if the closed loop efficiency of regulation is between 0 5 and 0 8 dB/dB

A similar calculation can be made on the basis of stimulus response curves plotted as function of sound pressure on a linear scale (in dyn/cm) (*cf* Fig 3) The attenuation during steady state is then obtained in dyn/cm as a function of sound pressure in dyn/cm (*cf* Fig 4) In the case regarded above with the closed loop efficiency of regulation in logarithmic units = 0 7 dB/dB the attenuation (dyn/cm) is very close to a straight line as a function of sound pressure (dyn/cm) with a slight upward concavity (increasing slope) It is well approximated by a straight line with

the slope 0 8 between 20 and 200 dyn/cm² The reflex can thus be assumed to regulate the input to the cochlea in a way equivalent to a subtraction of the muscle response from the envelope of the input sound At a sound pressure of 10 dyn/cm² above reflex threshold (approximately 20 dyn/cm²) the response of the closed loop system will have the same amplitude as the response of the open loop system at 2 dyn/cm above reflex threshold At this amplitude the attenuation is 8 dyn/cm The relation between attenuation (a measure of the feed back signal) and input sound pressure (actuating signal) for the open loop system will then have a slope of 8 0/2 0 = 4 0 above threshold for attenuation The ratio of the feed back signal to the actuating signal is the open loop transfer function The open loop gain for steady state can thus be estimated to 4 0 above threshold for attenuation The open loop efficiency of regulation and the open loop gain are equivalent concepts and under the present conditions approximately interconvertible

Discussion

Regulation of sound transmission through the middle ear

The total reflex In the present study it has been shown that pure tones in the frequency range from 0 5 kHz to 8 0 kHz were attenuated by the acoustic middle ear reflexes Low frequencies (0 5 kHz) were influenced by all degrees of middle ear muscle activity, whereas higher frequencies were attenuated only at levels well above the reflex threshold The magnitude and the frequency distribution of the effect strongly depended on level of muscle activity

Earlier quantitative studies on the regulation of sound transmission through the middle ear in mammals have not reported consistent results For example regulation of sound frequencies above 2 0 kHz has been observed by Bornshein and Krejci (1952) Gisselsson *et al* (1957) and Price (1966) whereas Wiggers (1937), Wever and Vernon (1955) Møller (1965) and Gerhardt and Wagner (1966) found no or very small influences above 1 5 to 2 0 kHz Anesthesia recordings with opened bulla and use of contralateral sound stimulation makes these mentioned data difficult to compare The discordance was probably to a great deal also due to the fact that the measurements in most cases were only made at one level of reflex activity

The efficiency of regulation was observed to be about 0 7 dB/dB *i e* if the sound pressure in the ear canal was increased 1 0 dB the middle ear reflexes attenuated the sound by 0 7 dB so that cochlear excitation increased by only 0 3 dB The efficiency of regulation of low frequency sounds in man is about the same (0 7 dB/dB Borg 1968) Wever and Vernon (1955) determined the efficiency of regulation in acute experiments on cats with open bulla They found that the closed loop efficiency of regulation was about 1 0 *i e* an increase in sound pressure in the ear canal resulted in no change in cochlear excitation This is not likely to be true throughout the dynamic range of the regulator (*cf* Borg 1968) but is more probably an effect of opening the bulla in the recording ear (Møller 1965 Borg 1972 a) The open loop efficiency of regulation *i e* the attenuation provided by middle ear muscle

contraction elicited by a sound not itself attenuated by the contraction is about 2 dB attenuation per 1 0 dB increase in sound pressure level The steady state open loop gain was estimated to 4 on the basis of the present data The high efficiency of regulation clearly shows that the middle ear reflexes are very potent regulators of the sound transmission

The relation between the m stapedius and the m tensor tympani reflexes The regulation of transmission of pure tones in the range investigated has been shown to be mainly provided by the m stapedius reflex. On the other hand the m tensor tympani has been found on a consistent basis to influence sounds at 0 5 and 1 45 kHz Since the excitability of the m tensor tympani reflex is lower than that of the m stapedius reflex (Wersall 1958 Borg 1972 c) it might function to extend the dynamic range of the total middle ear reflex activity The value of this increase for the regulation of sound transmission however is doubtful since the regulation provided by the m stapedius alone does not saturate even at levels far above that of the m tensor tympani threshold

Mechanisms for the attenuation of middle ear sound transmission Contraction of the middle ear muscles increases the stiffness component of the middle ear impedance (Møller 1965 Feldman and Zwislocki 1965), which in turn decreases the sound transmission through the middle ear below the resonance frequency of the middle ear However the increase in stiffness also increases the resonance frequency of the middle ear by about one half octave (from 1 5 to 2 2 kHz in the cat Møller 1965) and thus attenuation can be expected to occur up to about one half an octave above the resonance frequency of the ear with relaxed middle ear muscles

If that part of the middle ear which lies between the eardrum and the site where the m stapedius contraction increases stiffness (in the region of the stapes) is an analog to a series connection the magnitude of the impedance change of the ear and the change in transmission would be equal The results of experiments on cats and rabbits (Møller 1965) show that the change in transmission is larger than the change in impedance Above the resonance frequency (about 1 5 kHz) there is only a very small change in impedance On the other hand both in anesthetized (Gisselsson *et al* 1957 Price 1966) and in nonanesthetized animals (the present study) contractions of the middle ear muscles were found to attenuate the sound transmission several octaves above that frequency Furthermore the attenuation of the sound transmission in the frequency range investigated was found to increase even at sound levels well above that where the impedance change reached its maximum value (Borg 1968 1972 a the present study)

These results tend to indicate that a contraction of the m stapedius results in a functional decoupling of the cochlea from the middle ear in addition to the increase in stiffness The idea of decoupling was presented as an explanation to the experimental results of Møller (1961) and Mundie (1963) showing that the resistive part of the impedance (mainly due to the cochlea) was decreased at high levels of sound intensity (see also Feldman and Zwislocki 1965)

Dynamic properties

The origin of the oscillations The damped oscillations often seen in the response of the middle ear muscles to acoustic stimulation have been observed in many types of experiments (see e g Lorente de No 1935 Bornschein and Krejci 1952 Okamoto et al 1954 Eliasson and Gisselsson 1955 Wersäll 1958 Møller 1962) Several explanations have been suggested (a) proprioceptive mechanisms (Bornschein and Krejci 1952 Eliasson and Gisselsson 1955, Wersall 1958) (b) reverberant loops in the cochlear nuclei (Lorente de No 1935) or in higher centers (Baust and Berlucchi 1964 Salomon 1966) (c) regulation of sound transmission through the middle ear (Møller 1962) In the present study it was found that the oscillations in the contralateral m stapedius reflex disappeared after denervation of the m stapedius in the stimulus ear Thus these results strongly suggest that the oscillations originate in the m stapedius reflex feed back loop that serves to regulate sound transmission through the middle ear (cf conditions in man Møller 1962 Borg 1968)

The relation between the m stapedius and the m tensor tympani reflexes The information obtained in the present study allows only qualitative statements about the dynamic properties of the reflexes

Two features were observed that are not expected from a linear system (1) the rise time of the responses decreased as the stimulus intensity increased, (2) the rise time was in general smaller than the decay time Both of these nonlinearities were seen in the responses of the open loop as well as the closed loop systems and both were more prominent in the m stapedius reflex than in the m tensor tympani reflex (see Borg 1972 c) In spite of these nonlinearities it is of interest to note that the m stapedius reflex was faster than the m tensor tympani reflex in response to sound of equal intensity A qualitative estimate of this difference is obtained by comparing the resonance frequency of the closed loop responses of the isolated m stapedius and m tensor tympani reflexes As already pointed out (Borg 1972 c) they are about 18—22 Hz and 8—12 Hz respectively

The difference in the dynamic properties of the two regulators working in parallel may improve the stability of the total feed back loop regulating the sound transmission through the middle ear This hypothesis is supported by the observation that cutting the m tensor tympani increased the magnitude of the oscillations in the closed loop response (Borg 1972 c) and in model experiments (Borg 1972 e) Furthermore the two types of nonlinearities observed have been found to improve feed back loop stability in model experiments (Borg 1972 e)

One would expect that maximal speed of regulation of sound transmission would be desirable It does not seem to be neural or mechanical factors that limit the speed of the middle ear reflex responses In man the oscillation frequency of the m stapedius response is 5 Hz (Møller 1962) whereas the frequency of finger tremor is 8—12 Hz (Lippold 1970 The significance of the dynamic properties can however only be judged in relation to such properties of the auditory system as the time course of psychoacoustic processes and the development of damage to the cochlea

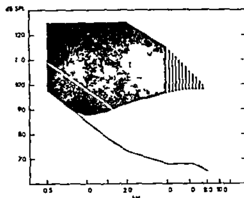


Fig 7 Summary of acoustic middle ear reflex properties Threshold of total reflex (m stapedius in conjunction with m tensor tympani) is shown by the thin continuous line and it is identical to m stapedius threshold Threshold of m tensor tympani is shown by thin white line Dark shaded area shows region where single pure tones are attenuated by the middle ear muscle contraction elicited by the pure tone itself The upper limit of the dark shaded area approximately represents the limit of available sound pressure level

Functional role of the acoustic middle ear reflexes in the auditory system

The functional characteristics of the acoustic middle ear reflexes in the nonanesthetized rabbit are summarized in Fig 7 on the basis of results of the present study and results presented by Borg (1972 c) The thin continuous line shows the threshold (defined as 10 % of maximal impedance change to ipsilateral stimulation) of the total reflex (*i e* identical to the threshold of the m stapedius) and the white line shows that of the m tensor tympani The heavy continuous line and its dotted extension shows the threshold for attenuation of sound transmission through the middle ear

On the basis of these results it is not possible to directly describe the function of the middle ear reflexes in psychoacoustic terms It is possible however to relate the function of the middle ear reflexes to certain basic concepts in sensory physiology *e g* dynamic range lateral inhibition and adaptation

Dynamic range for intensity means the range of sound pressure level in the ear canal for which the ear is operating satisfactorily The effect of over loading might be to decrease the discrimination ability or to induce fatigue or damage to the ear As is shown in Fig 7 sound in the entire frequency range investigated is attenuated above 90 to 100 dB SPL. If noisebands are used and if both ears are stimulated simultaneously the threshold for influence on sound transmission is expected to be lowered by 10—20 dB as compared to the unilateral pure tone stimulation as used in the present study (Møller 1962 Flottorp *et al* 1971) As consequence of the attenuation the slope of the stimulus response curve of the cochlea is decreased (see Fig 3) and thus the dynamic range is increased due to the reflex activity Since the total dynamic range of the ear is less at low frequencies (0.5 kHz) than at high (4.0 kHz) this effect might be more important in the low frequency range The functional consequence of this compression is that the stimulus response curve of the cochlea *below* the reflex threshold can be steeper with than without feed back regulation and discrimination might be better In case that extension of the dynamic range is the most important function of these reflexes it is to be expected that the

reflex thresholds should be above the sound intensity range most significant for the animal It might also be important that one type of nonlinearity in cochlear function appears at 80—90 dB SPL (Worthington and Dallos 1971)

It should be noted in this connection that the olivocochlear efferents influence the excitability of the cochlea mainly for low intensities in the medium and high frequency range (Wiederhold 1970 Borg 1971) The result of that is also an increase in the slope of the stimulus response curve of the cochlea at low intensity Detailed knowledge about the functional and neuroanatomical relation of the middle ear reflexes to the olivocochlear efferents is however sparse (see however a recent study by Desmedt *et al* 1971)

As shown in Fig 7 low frequency sound is attenuated by all degrees of middle ear muscle activity, whereas high frequencies are attenuated only far above reflex threshold (dark shaded area) In addition high frequency sound is the most effective in eliciting reflex responses (light shaded area of Fig 7) Consequently in a sound with energy in a wide frequency range (partly within the light shaded area of Fig 7), the low frequency components will be attenuated by the reflex activity elicited by the high frequencies an effect which can be described as lateral inhibition The high frequencies are not influenced unless the reflex activity is close to maximum In addition to the feed back attenuation (a sound is attenuated by the reflex elicited by the sound itself) there is an effect similar to lateral inhibition by which a high frequency sound attenuates a sound of low frequency

In psychoacoustic experiments it has been shown that low frequency sound serves to mask the perception of high frequencies The masking effect is very extensive at high intensity of the masking sound (Ehmer 1959) An attenuation of the low frequencies is thus expected in many cases to be followed by an improvement in perception of high frequencies For example a reduction in the intensity of a 0 5 kHz masking tone from 80 to 60 dB (sensation level) is followed by a 70 per cent decrease of masking expressed as the area in the log frequency intensity diagram (data from Ehmer 1959) Thus the acoustic middle ear reflexes have a function similar to lateral inhibition by which the masking effect of low frequencies on high frequencies is decreased

Adaptation is often defined as a decrease in the sensitivity of a system following activity in the system (see *e g* Harth *et al* 1970) The middle ear reflexes are activated by sound and their activity decreases the response of the cochlea with some time lag (for details on the dynamic properties see Borg 1972 d) Thus the middle ear reflexes result in adaptation of cochlear response Thereby they decrease the response to continuous stimuli to a greater degree than to time varying stimuli and thus changes in sound intensity may be enhanced It is of interest to note that model experiments have shown that adaptation properties in neurons can improve signal transmission in neuronal chains under certain circumstances (Harth *et al* 1970)

In conclusion the acoustic middle ear reflexes have functions which can be described as increasing dynamic range lateral inhibition and adaptation

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Production and Removal of Lactate during Exercise in Man

By

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Abstract

HERMANSEN L and I STENSVOLD *Production and removal of lactate during exercise in man* Acta physiol scand 1972 *86* 191—201

Blood lactate concentration (LA) was measured in 4 female and 3 male well trained subjects before and during 30 min of continuous treadmill running at 4 different speeds demanding about 30 60 70 and 80 % of the individuals maximal oxygen uptake ($\dot{V}_{O_2}$ max) The same subjects also performed in another series of experiments where maximal intermittent exercise preceded 30 min of running at the same 4 speeds or resting in a chair During continuous running starting from resting conditions the blood LA increased only slightly up to a critical level (i e 60—80 %) of $\dot{V}_{O_2}$ max From then on a pronounced lactate production may occur During the maximal intermittent exercise blood LA increased to 130—270 mg/100 ml In the recovery period i e continuous running at the same 4 speeds or resting in a chair blood LA decreased towards resting values The lactate removal rate was calculated from the rectilinear part of the curves describing the changes in LA with time and expressed as mg/100 ml × min The lactate removal rate was higher during exercise than during rest and increased with increasing work load up to the same critical level (i e 60—80 % of $\dot{V}_{O_2}$ max) beyond which a reduction was observed The highest removal rate was 8 mg lactate/100 ml × min at 63 % of $\dot{V}_{O_2}$ max (average values) These results indicate that human skeletal muscle possesses a pronounced capacity to oxidize lactate Therefore a production of lactate is possible even with no increase in the blood LA These results also indicate that the skeletal muscle rather than the liver may be regarded as the main site for lactate removal during exercise

It is well known that the blood lactate concentration is increased during maximal exercise (Hermansen 1971) Several investigations have shown an increased blood lactate concentration also during submaximal exercise (Hill and Lupton 1923 Bang 1936 Hermansen and Saltin 1967) According to Margaria and co workers (Margaria *et al* 1963 Saiki Margaria and Cuttica 1967) no lactate is produced once a steady state of oxygen uptake is reached even at work loads close to the individual maximal oxygen uptake However several investigations (Bang 1936 Karlsson and Saltin 1970 Nagle *et al* 1970) have shown that the lactate concentration in both muscle and blood increased gradually during the course of a submaximal work load lasting for 15 min or more Thus the significance of lactate production in energy metabolism during submaximal exercise is yet not clear

TABLE I Data characterizing the subjects

Subj	Sex	Age yr	Height cm	Weight kg	Maximal Oxygen Uptake ml/kg × min
A G	F	21	171	67	49 7
R G	F	28	169	57	52 4
K H	F	26	168	59	53 4
G S	F	21	173	70	49 5
E E	M	24	187	80	64 2
J H	M	28	170	64	73 1
U R	M	26	184	66	69 2

In 1928 Jervell showed that the blood lactate concentration could be made to fall faster compared with resting conditions if moderate exercise was performed in the recovery period This observation was later verified by several other investigations (Bang 1936 Newman *et al* 1937 Gisolfi Robinson and Turrel 1966 Rowell *et al* 1966 Davies Knibbs and Musgrove 1970) However the relationship between the rate of lactate removal and the severity of the work load performed in the recovery period is not yet settled

The aim of the present investigation was to elucidate further whether or not lactate is produced during submaximal work and furthermore to study the rate of lactate removal in relation to different metabolic activities in the recovery period

Materials, methods and procedure

4 female and 3 male well trained subjects participated in the study Pertinent data describing the subjects are given in Table I

Oxygen uptake was determined by the Douglas bag technique using a low resistance valve The volume of the expired air was measured in a spirometer and the gas analyses were carried out with the Scholander apparatus (Scholander 1947) Blood lactate concentrations were analyzed according to the Strom modification (Strom 1949) of the colorimetric method of Barker and Summerson (1941)

Oxygen uptake was measured during at least 3 submaximal and 2 maximal work loads on 2 or 3 different days during the pretest period In general the procedure suggested by Hermansen and Saltin (1969) was used in all determinations of the maximal oxygen uptake All work experiments were performed on a motordriven treadmill set at an inclination of 3 (5 25) In the rest experiment the subjects were placed in a comfortable chair

After this pretest period all subjects came to the laboratory on 9 different days On the first 4 days the subject performed 30 min of continuous walking or running after having rested for 20—30 min in the laboratory The speeds were chosen according to the results from the pretest period and represented about 30 60 70 and 80 % of the individual's maximal oxygen uptake

On the next 4 experimental days the subjects performed a maximal intermittent exercise program directly followed by 30 min of continuous treadmill walking or running at the same work loads as described above i e about 30 60 70 and 80 of the individual's maximal oxygen uptake The maximal intermittent exercise consisted of 3 maximal work bouts each lasting for about 60 The subjects ran at their highest possible speed (i e 290–310 m/min for the female and 360–380 m/min for the male subjects) with a rest period of approximately 4 min in between On the last 9th experimental day the subjects rested in a comfortable chair for 30 min after the maximal intermittent exercise program had been terminated Blood samples were taken from a prewarmed clean and dry finger at different time intervals before and during the exercise program and during the rest experiments

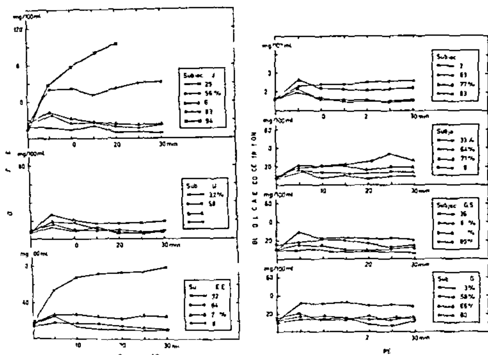


Fig 1 Individual values for the blood lactate concentration during continuous treadmill running at different speeds for 30 min

Results

Individual values for blood lactate concentration during continuous treadmill walking or running at the 4 work loads representing approximately 30 60 70 and 80 % of the individuals maximal oxygen uptake are given in Fig 1 No significant increase in the blood lactate concentration was found when the subjects performed at the 2 lowest work loads (i e about 30 and 60 % of the individuals maximal oxygen uptake) Even at the 2 highest submaximal work loads demanding approximately 70—80 % or more of the individuals maximal oxygen uptake only a slight increase (i e up to 40 mg/100 ml) in the blood lactate concentration could be demonstrated Only two subjects J H and L E showed a pronounced increase at the highest work levels These level represented an oxygen uptake of 62 9 and 71 6 ml/kg×min for subject J H and 53 6 ml/kg×min for subject E E

Fig 2 gives the individual values for the blood lactate concentration (i e the values obtained after 10 min of work) in relation to the relative work load i e oxygen uptake in per cent of maximal oxygen uptake It should be noted that no increase in the blood lactate concentration could be observed up to a work level representing approximately 60—70 % of the individuals maximal oxygen uptake However when this work level is passed the blood lactate concentration is increasing very rapidly in some subjects while others show almost no increase at all

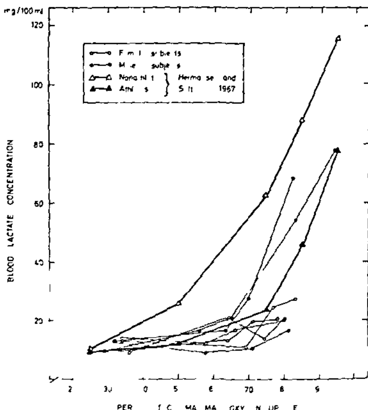
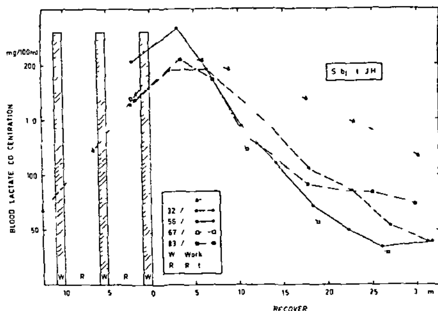


Fig. 2. Individual values for blood lactate concentration (i.e. the value obtained after 10 min of exercise) in relation to relative work load. The mean values for trained and untrained subjects (Hermansen and Saltin 1967) are included for comparison.

In Fig. 3 are illustrated the results obtained for the blood lactate concentration in relation to time for 2 representative subjects from the experiments where the maximal intermittent exercise preceded the continuous submaximal work. In all subjects the blood lactate concentration was found to increase from about 10 mg/100 ml at rest to 130–220 mg/100 ml (average 165 mg/100 ml) after the last (third) maximal exercise bout. During the recovery period which consisted of rest (i.e. sitting on a chair) or exercise (i.e. continuous walking or running at different speeds on the treadmill) the blood lactate concentration decreased toward resting value.

The rate of lactate removal (i.e. the net removal) was calculated from the slope of the rectilinear part of the curves describing the changes in blood lactate concentration in relation to time (i.e. between approximately the 5th and the 15th min of the recovery period). The rate of removal was expressed as mg lactate per 100 ml of blood and min (i.e. mg/100 ml/min). It should be noted (Fig. 3) that the rate of lactate removal was markedly higher when the subjects were performing exercise than when resting in the recovery period. The rate of lactate removal was also affected by the intensity of the work load. Individual values for the rate of lactate



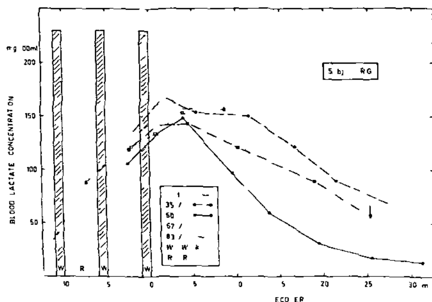


Fig 3 Blood lactate conc ntration in relation to tin fo ? of the / ubjects (befo e) during and after 3 maximal exerci e bouts to exhaustion The reco ry period consisted of rest (*i e* sitting in a chair) or walking ɔr running on the treadi nll at d ff re it speeds

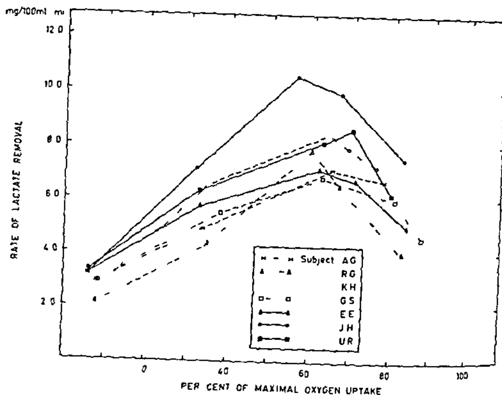


Fig 4 Rate of lactate removal (i e net removal) in relation to relative work load

removal in relation to relative work load (i e oxygen uptake expressed in per cent of maximal oxygen uptake) are given in Fig 4 The rate of lactate removal calculated according to the method described above increased with increasing relative work load up to a critical work level representing 60—70 per cent of the individual maximal oxygen uptake The average maximal rate of lactate removal was found to be 8 mg/100 ml × min at 63 per cent (mean values) of the individuals maximal oxygen uptake Beyond this critical work level (i e 60—70 % of maximal oxygen uptake) a pronounced decrease in the removal rate was observed in all subjects However the rate of lactate removal was on the average significantly higher at the highest work load (i e approximately 80 % of maximal oxygen uptake) than at rest

Thus a work load representing 60—80 % of the individuals maximal oxygen uptake seems to be a critical level both for lactate production and lactate removal

Discussion

It has been known for many years that the blood lactate concentration is increased as a result of muscular exercise (Hill and Lupton 1923 Jervell 1928 Margaria Edwards and Dill 1933 Bang 1936) However the significance of lactate production

during submaximal exercise is still a subject of dispute (Saiki Margaria and Cuttica 1967, Nagle et al 1970) According to Margaria and co workers (Margaria et al 1963 Saiki et al 1967) no lactate production occurs during submaximal exercise once a steady state of oxygen uptake is reached The increase in blood lactate concentration observed during submaximal work is according to these authors limited to the first phase of the exercise period during which the oxygen dept is contracted On the other hand several studies have shown that the lactate concentration in both muscle and blood increased with time during prolonged submaximal work (Nagle et al 1970 Karlsson and Saltin 1970)

In the present investigation most of the subjects did not show any marked elevation of the blood lactate concentration during continuous running although the work loads ranged between 30 and 90 % of the individuals maximal oxygen uptake This observation is in conflict with the results from several other investigations (Bang 1936 Wyndham et al 1965 Williams et al 1968) using untrained subjects and bicycle exercise However our results are substantially in agreement with results obtained during treadmill running (Saiki et al 1967 Costill et al 1971) Studies by Hermansen and Saltin (1969) showed that the blood lactate concentration is lower during submaximal treadmill exercise than during bicycle exercise at the same metabolic rate Similar results have been obtained when lactate production during exercise with large muscle groups (i e leg exercise) and small muscle groups (arm exercise) is compared (Asmussen and Nielsen 1946 Sternberg et al 1967) Furthermore it was shown by Hermansen and Saltin (1967) that well trained subjects elicited lower blood lactate concentrations than untrained subjects not only at the same absolute work load but also at the same relative work load (i e the same per cent of maximal oxygen uptake) Thus the discrepancy between the values for blood lactate concentration during submaximal exercise in the present study and those of many others may be at least partly explained by differences in the methods employed and the material examined

A major question in the present study was whether or not lactate is produced during submaximal exercise However it should be emphasized that when measurements of the blood lactate concentration alone are used the possibilities for answering this question are limited Under conditions where an imbalance between production and removal exists the rate of net production or net elimination of lactate may be calculated However under conditions where lactate concentration is kept at a constant level it is impossible to tell from measurements of the concentration only whether or not lactate is produced To solve this problem both turnover studies with C^{14} labelled lactic acid (Depocas Minaire and Chatonnet 1969 Jorfeldt 1970) and measurements of arterio venous lactate difference (Stainsby and Welch 1966 Keul Doll and Keppler 1969) have been used However these studies have yielded conflicting results

Another approach which is used in the present investigation is to study whether or not a capacity to eliminate lactate during exercise is present and furthermore whether or not the capacity to eliminate lactate is large enough to cope

expected lactate production during exercise. From the results of the present investigation (Fig. 3 and 4) it is seen that the rate of lactate removal is greatly enhanced with increasing work load up to some critical level representing approximately 60—70 % of the individuals' maximal oxygen uptake. The average removal rate was 2.9 mg/100 ml×min at rest and 8.0 mg/100 ml×min at a work load representing 63 % of the individuals' maximal oxygen uptake. A further increase of the work load, however, produced a pronounced decrease in the rate of lactate removal.—However, it should be emphasized that an appreciable amount of lactate is removed even at work loads demanding 80—90 % of the individuals' maximal oxygen uptake.

The observation that the rate of lactate removal is facilitated by the performance of exercise in the recovery period is not new (Jervell 1928, Newman *et al.* 1937, Rammal and Strom 1949, Gisolfi, Robinson and Turrell 1966, Rowell *et al.* 1966, Davies, Knibbs and Musgrove 1970). However, the fate of the removed lactate is yet not settled.

In seeking the explanations for the increased rate of lactate removal several factors are to be considered. The importance of the liver in the elimination of lactate during exercise is pointed out by several investigations (Rowell *et al.* 1966, Davies *et al.* 1970). In addition to the liver, skeletal muscle (Jorfeldt 1970) and other organs (for references see Knuttgen 1971) are able to remove lactate even during the course of muscular work.

If one supposes that lactate, which is a rather small and easily diffusible molecule, is evenly distributed in the extracellular and intracellular water, it is possible to calculate the approximate amount of lactate produced in the body or removed from it. For the purposes of these calculations it was assumed that the fraction of water was 0.6 for the body and 0.8 for the blood, as proposed by Margaria *et al.* (1963). In the present investigations intermittent maximal exercise of short duration increased the blood lactate concentration to approximately 150 to 200 mg/100 ml representing a total net production of approximately 70—100 grams. With regard to the net removal of lactate in the recovery period 2.9 and 8.0 mg/100 ml were eliminated each minute at rest and during exercise (63 % of the individuals' maximal oxygen uptake), representing approximately 1.0—1.5 g per min at rest and 4.0—4.5 g per min during work.

According to the studies of Rowell *et al.* (1966) approximately 50 % of the total amount of lactate eliminated was removed by the liver, indicating that the liver was the main site of lactate removal during exercise. However, both the blood lactate concentration and the total amount of lactate removed per minute was much lower than in the present study. Using the values for liver blood flow and the highest a-v lactate difference given by Rowell (1971), the total amount of lactate removed was approximately 150 mg/min (*i.e.* about 3—4 % of the lactate removed in the present study). Thus, if these calculations are correct, the importance of the liver in the elimination of lactate during exercise may be regarded as insignificant.

In making these calculations the value for the a-v lactate difference in the liver

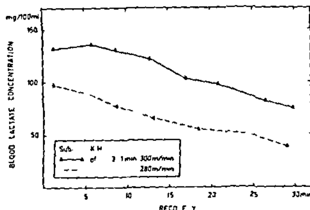


Fig 5 Blood lactate concentration for one subject in the recovery period after 3 maximal work bouts of different intensity (i e 300 m/min upper curve and 280 m/min lower curve)

(and the splanchnic organs) is of primary importance since large variations in the splanchnic blood flow during exercise are not to be expected (Rowell 1971) Hence the crucial question is to what extent the arterio venous lactate difference is influenced by the magnitude of the arterial blood lactate concentration

In the studies of Rowell *et al* (1966) it is indicated that the rate of lactate uptake in the splanchnic organs was proportional to the arterial blood lactate concentration Thus the validity of our calculations might be questioned since the arterial blood lactate concentrations in the present study were approximately 5—6 times higher than in the studies of Rowell *et al* (1966) However as can be seen from the results in Fig 5 fairly large variations in the capillary blood lactate concentration did not alter the rate of lactate removal This observation may possibly be explained by assuming an increase in the rate of lactate uptake with increasing arterial blood lactate concentration up to some critical value (i e 40—50 mg/100 ml) large enough to give the maximal enzymatic rate in the liver cells Hence if these assumptions are correct the rate of lactate removal by the splanchnic organs is not significantly influenced by the arterial blood lactate concentration once the critical value is passed Thus according to these assumptions the liver is not able to cope with more than a small fraction of the total lactate removed during exercise

Lactate is to a certain extent eliminated in sweat and urine (Knuttgen 1971) Furthermore it is also known that lactate is metabolized in myocardium and resting skeletal muscle (Rowell 1971) However the significance of the working skeletal muscles in the removal of lactate during exercise has so far been neglected The skeletal muscles constitute the largest organ of the body representing approximately 40 % of the body weight (Andres Cader and Zierler 1956) Moreover recent studies by Jorfeldt (1970) and Karlsson (1971) have indicated that the exchange of lactate between muscle and blood is a rapid process Furthermore it is a well known fact that the splanchnic blood flow is reduced markedly during exercise A similar reduc

tion is observed in other organs as well (Rowell *et al* 1966) On the other hand the blood flow through the working skeletal muscle is increased and the turn-over of metabolic substrates is high (Karlsson 1971) All of these observations obviously offer a conceivable interpretation of the pronounced lactate removal during exercise observed in the present investigation Thus the results of the present investigation seem to indicate that the oxidation of lactate in the working muscles is the preferred pathway rather than oxidation or gluconeogenesis in other tissues

The present investigation failed to give any definite answer to the question of whether or not lactate is produced during submaximal work load demanding up to 60—80 % of the individuals maximal oxygen uptake However it was shown that the human skeletal muscle possesses a pronounced capacity to oxidize lactate indicating that this possibility is present Our results support earlier studies showing that a work load of 60—80 % of the individuals maximal oxygen uptake represents a critical level beyond which a pronounced increase in the lactate production may occur

Finally the results of the present investigation indicate that the human skeletal muscle rather than the liver may be regarded as the main site for lactate removal during exercise

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Acta physiol scand 1972 86 202—210
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Low Flow States in the Microvessels of Skeletal Muscle in Cat

By

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Abstract

Eriksson E and B Lisander *Low flow states in the microvessels of skeletal muscle in cat* Acta physiol scand *1972 86 202—210*

With the tenuissimus muscle exposed for vital microscopy the effects on macro and microcirculation of aortic compression and of arterial bleeding were studied in cats anesthetized with chloralose In the first case arterial pressure in the hindlimbs was kept at 50 mm Hg for 2 1/2 h and in the latter the animal was rapidly bled with 40 % of its total volume and then observed for up to 4 h — During aortic compression arterioles of 25 μm internal diameter increased their dimensions with about 40 % and arteries of 70 μm with about 15 % The diameters of the venules showed a slight decrease Linear flow rates in the capillaries decreased but there was an even distribution of the blood flow No formation of thrombi was seen — At arterial bleeding all the vessels first reduced their diameters during 10—20 min Then arterioles of 25 μm diameter dilated while the diameters of the other vessels remained reduced In average the cats survived for 2 1/2 h Within a few minutes after the bleeding there was an adhesion of white cells to the venular walls and in later stages aggregates of red cell platelets and white cells started to occlude most venules and capillaries leading to extensive functional shunting" at the capillary level

A large blood loss inevitably leads to a decreased systemic arterial blood pressure while the peripheral circulation is influenced also by changes in vascular tone and by alterations in the flow properties of blood Except for the recent study by Bieckstrom *et al* (1970) there appears to have been no attempt using a functional approach to differentiate *in vivo* between the effects caused by these latter two factors Intravital microscopy has however been applied to several vascular beds and it offers the possibility of measuring vascular dimensions and observing the behaviour of blood in the microvessels of *non heparinized* animals Skeletal muscle tissue constitutes 30—40 per cent of the cat s lean body mass but due to methodological difficulties there are few reports dealing with direct quantitative observations of its vascular bed in connection with hemorrhage Such a study may however provide a better understanding of the overall consequences of a blood loss and for this purpose the transilluminated tenuissimus muscle has been utilized in the present experiments A preliminary report has been presented (Brånemark Eriksson and Lisander 1971)

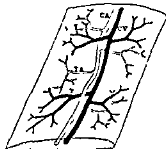


Fig 1 Schematic representation of the vascular arrangement in the tenuissimus muscle CA = central artery CV = central vein TA = transverse arteriole and TV = transverse venule

Methods

The study was performed on 34 cats anesthetized with chloralose (50 mg/kg b w) following induction with ether A tracheal cannula was inserted and body temperature was maintained constant with a heating pad The cats were neither heparinized nor given any substitutional fluid during the experiment

The left tenuissimus muscle was exposed for observation in transmitted light according to the method of Brånemark and Eriksson (1972) The muscle was observed under the microscope in ordinary mixed white light from which the infra red component had been filtered out A green filter with maximum transmission at 5500 Å was used to minimize damage to the observed tissue About 1 cm of the muscle was observed during the experiment. Objectives (×4 NA 0 15 ×10 NA 0.30 ×23 NA 0.55 ×55 NA 0 84 ×75 NA 1 00) giving magnifications from 40 to 750 times were used Internal vessel diameters were measured with an optical micrometer Linear flow rates were calculated with a flying spot method (Brånemark and Jonson 1963) Photographic and videotape recordings were made

Siliconized saline filled cannulas were inserted in the left carotid artery in the femoral artery and in the left jugular vein the latter cannula with its tip in or close to the right atrium The pressures in the carotid and the femoral arteries were recorded *via* transducers (Statham P 23 AC) recording on a Grass polygraph The central venous pressure was followed using a water manometer PO_2 PCO_2 and pH were measured in arterial blood samples *

In all animals the abdomen was opened in the midline The catecholamine secretion from the adrenal medullae was eliminated in some experiments by ligation of the right and surgical denervation of the left adrenal In this way adrenorcortical function was maintained on one side When interruption of the sympathetic nerve supply to the tenuissimus muscle was to be performed a thread was placed around the ipsilateral lumbar sympathetic chain at L4—L5 and pulled during simultaneous observation of the vessels

The cats were subdivided into 2 groups In the first group the aorta was compressed by a screw-clamp at the lumbar level decreasing the mean blood pressure in the femoral artery to about 50 mm Hg This pressure was maintained for 2 1/2 hours after which the compression was released Following this the muscle was observed for another 2 h The second group was bled rapidly corresponding to 35—40 per cent of the total blood volume on the assumption that total blood volume in the cat is 6 per cent of the body weight (Farnsworth Paulino-Gonzalez and Gregersen 1960) With this large and rapid bleeding the mean blood pressure fell transiently to about 30 mm Hg and stabilized after some ten minutes at around 80 mm Hg The animals were observed for a maximum of 6 h At the end of the experiment biopsies from *the observed left and from the control tenuissimus muscle of the right limb were* taken for histological and ultrastructural analyses

Results

The general vascular architecture in the tenuissimus muscle in the cat has been described in detail in another publication (Eriksson and Myrhage 1972) Fig 1 shows the principal vascular arrangement which is very regular There is one central artery with an internal diameter of about 70 μm and one central vein with an in

* These measurements were performed with the kind help of Dr Egil Haggendal Dept of Clinical Physiology University of Gothenburg Gothenburg

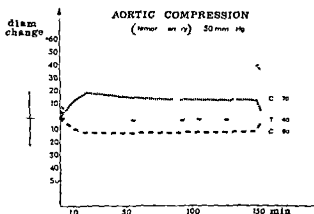


Fig 2 Changes in internal diameters of the vessels in the tenuissimus muscle in the cat during aortic compression For explanation of the symbols of the vessels see Fig 1 Femoral artery pressure is reduced to 50 mm Hg The occlusion period begins at zero time and ends at 150 min The diagram represents the findings in one experiment in a cat with intact sympathetic chain and adrenals This diagram is almost identical to the compiled data from the 6 cats in this group of animals The calculated standard deviation is of the order of 20 %

ternal diameter of about 90 μm These vessels run parallel to the muscle fibres The transverse arterioles have internal diameters of about 25 μm and the transverse venules of about 40 μm These vessels branch off at right angles from the central artery and vein respectively Via a series of branches the transverse vessels supply the capillaries which again run parallel to the muscle fibres This microvascular bed was directly studied in two types of experiments

In the first type aortic compression was performed in such a manner as to maintain the pressure in the femoral artery at 50 mm Hg for 2 1/2 h This resulted in a dilation of precapillary vessels (autoregulation) which was most pronounced in the transverse arterioles (Fig 2) The central arteries dilated by about 15 % while the transverse arterioles dilated by as much as 40—45 % The diameters of the venules often varied drastically due to collapse of their lumina When this did not occur there appeared to be a slight decrease in their diameters The diameters of the capillaries remained constant throughout the experiment Linear flow rates decreased markedly but tissue perfusion remained uniform throughout the observation period

The erythrocytes sometimes formed stagnant masses in the microvessels usually in the venules where they formed distinct rouleaux This type of red cell aggregation did not appear to have significant mechanical stability as the rouleaux could be broken apart by even small increases in the linear flow rate or by occasional fasciculations in the preparation When the aortic compression was released linear flow rates and vascular diameters returned to pre compression values within one minute The vascular luminal adjustments during or following this type of pressure decrease was largely the same when the vascular bed had previously been autonomically denervated or when the adrenal medullae had been excluded and also when both these procedures were combined

In the second type of experiment when the effects of hemorrhage were to be studied the cats were bled rapidly by about 40 % of their estimated blood volume These animals survived the blood loss for an average of 2 1/2 h Data on one animal

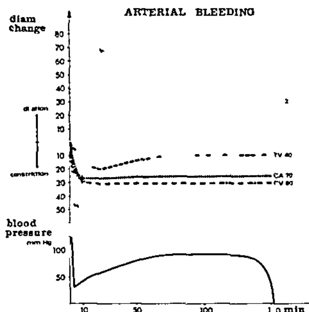


Fig 3 Changes in internal diameters of the vessels in the tenuissimus muscle in the cat after arterial bleeding At zero time the bleeding starts and during 2—5 min about 40 % of the total blood volume is removed For explanation of the symbols of the vessels see Fig 1 The diagram shows the results from 1 exp where the sympathetic chain and the adrenals were left intact The compiled data from the 6 cats in this group show similar results The calculated standard deviation is of the order of 30 %

with the sympathetic nerves and the adrenals intact bled to the same extent and to about the same pressure level is shown in Fig 3 During the first five minutes after the blood loss there was a decrease in the diameters of all vessels but then a dilation of the transverse arterioles occurred The vascular diameters stabilized at a constant level after about 60 min As in the animals with aortic compression the veins also now showed a marked tendency to collapse but this occurred at a higher arterial pressure suggesting a larger pressure drop along the precapillary vessels Except for this the reactions of the venous vessels after bleeding were very similar to those seen during aortic compression Often blood cells were trapped in the collapsing lumina

Cats which had undergone either regional sympathetic denervation of the studied limb or exclusion of the adrenal medullary secretion displayed luminal changes that were qualitatively and quantitatively similar to those of intact preparations If however both the adrenal medullae were excluded and the local sympathetic nerves cut arterioles now displayed a dilation after only 30—60 s without any preceding constriction while the luminal changes of other vessels in the tenuissimus muscle were quantitatively largely the same as in the innervated preparation Surprisingly a small diameter decrease of the central arteries could still be observed and was of about the same magnitude as in the animal shown in Fig 3 in which both the sympathetic nerves and the adrenal meduallary secretion had been preserved

During the whole observation time after bleeding there was an adhesion of the white cells to the walls of the venules (Fig 4) After about 20 min some of these cells migrated through the vessel walls and increasing numbers could be found out

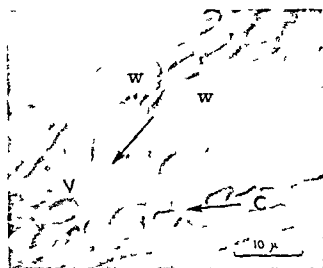


Fig 1 White cells (W) adhering to the walls of a venule (V) in the tenuissimus muscle after arterial bleeding These cells decrease the lumen of the venule and thus add to the vascular resistance of this part of the vessel The internal diameter of the venule is 15 μm The deformability of the red cells is visualized in the capillary (C)

ide the vessels Histological examination of the perivascular regions revealed that these cells were polymorphonuclears mostly neutrophils

About 20 min before the animals died the venules started to be totally filled with clusters of red cells platelets white cells and fibrin The presence of fibrin was shown at analysis of specimens from these muscles in the electron microscope (Ericson and Eriksson 1972 to be published)

These clusters were first observed in venules of about 20 μm and later extended both upstream and downstream Within a few minutes after the appearance of the first clusters some of the venules were occluded and after 10—15 min there was a total standstill in all the vessels within the muscle The cells appeared to adhere firmly both to each other and to the vascular walls In the central vein they were occasionally washed away by the blood stream creating microemboli but in most vessels this did not take place A microthrombus formation occurred also in the regionally sympathectomized muscle preparations However it should be stressed that such thrombi were not observed in cats in whom the adrenal medullary secretion had been excluded before the blood loss Here the blood remained fluid in all microvessels observed In the bled animals there was an uneven distribution of flow as soon as the thrombosis occurred This was in contrast to cats in whom aortic compression was performed in those animals tissue perfusion always remained uniform even at femoral artery pressures as low as 40 mm Hg Capillaries from the distal parts of the transverse arterioles became blocked in the bled animal while the most proximal capillaries usually remained unobstructed Grossly uneven capillary perfusion did not occur until microthrombotization was evident In some of the bled cats PO_2 PCO_2 and pH were followed in arterial blood PCO_2 remained fairly stable in these anaesthetized animals while pH showed a slight decrease after bleeding accelerating terminally PO_2 was fairly constant up to 30 minutes before the cats died when it showed a decrease

Discussion

A large blood loss results in a reflex constriction of resistance vessels as well as of capacitance vessels This tends to raise central arterial and venous pressures and also favours absorption of fluid from the interstitial phase Subsequently there is a secondary relaxation of the resistance vessels due to an overpowering of neurogenic vasoconstriction by local dilator factors (for ref see Chien 1967 and Mellander and Johansson 1968)

Considerable changes in the flow properties of blood also occur following hemorrhage but there often seems to be little correlation between the rheological properties of blood *in vitro* and *in vivo* A widespread microplugging and erythrocyte trapping in the peripheral vessels may thus occur in a way that tends to lower blood viscosity as measured *in vitro* while causing an apparent *increase* of blood viscosity *in vivo* as a result of the mentioned microplugging of the biological viscometer (Baeckström *et al* 1970) Hemorrhage may further lead to widespread intravascular coagulation causing deposition of fibrin and additional trapping of blood cells in the microvessels (see Hardaway 1966) an effect that for natural reasons is largely absent in many *in vivo* studies utilizing techniques calling for inactivation of the clotting mechanisms

Intravital microscopy offers the possibility of measuring vascular dimensions and at the same time observing gross changes in the behaviour of blood in the microvessels In the present study neither heparinization nor fluid substitution were performed

In some animals the aorta was compressed at the lumbar level decreasing blood pressure in the femoral artery to about 50 mm Hg so as to cause regional hypotension in the tenuissimus vascular bed studied This led to a dilation of the precapillary vessels which was most pronounced in the transverse arterioles Since the geometry and dimensions of the venous vessels are known to vary markedly with changes in transmural pressure (Öberg 1967) no definite conclusions are warranted as to active changes in venular tone In the experiments in which partial aortic occlusion was induced the passive and active luminal changes were probably due solely to regional alterations in transmural pressure and to secondary changes of the local chemical environment This assumption is corroborated by the fact that no differences in the luminal adjustments were observed if the regional sympathetic nerves had been cut or if the adrenal medullary secretion had been excluded The capillaries did never collapse even when the femoral artery pressure was as low as 15—20 mm Hg contrary to the findings of Algire (1954)

It remains unclear whether the widespread venous collapse as observed in the present preparation facilitated the microvascular thrombotization and in turn imposed an increased resistance to flow It is evident though that the collapse may elicit a circulatory standstill in some parts of the vascular network and also cause a trapping of blood cell

There was an adhesion of white cells to the walls of the venules during the whole observation period after the bleeding with some of these cells mainly polymor

phonuclear neutrophils migrating through the vessel walls after 20 min (Ericson and Eriksson, to be published) 20 to 30 min before death which occurred on the average 2 1/2 h after bleeding a large fraction of the venular lumina became completely filled with masses of red cells platelets white cells and fibrin This occurred first in venules of about 20 μm size and then extended into capillaries but not arterioles Within 5 min a lot of the venules were occluded

It is well known that following bleeding the activity in the sympathetic vasoconstrictor fibres to the muscles increases markedly (see *e g* Öberg 1964), but the microthrombotization was not affected by regional sympathectomy On the other hand exclusion of the adrenals appeared to abolish this phenomenon in agreement with the findings by Baeckstrom *et al* (1970) It is well known that splanchnic nerve stimulation may increase the coagulability of blood an effect that is abolished by adrenal extirpation (Cannon and Mendenhall 1914) Robb (1963) found an increased platelet aggregation after i v infusion of noradrenaline and many investigations have now shown that platelet aggregation can be precipitated by catecholamines both *in vitro* and *in vivo* particularly by adrenaline effects that are blocked by adrenergic receptor blocking agents (Bygdeman and Johnsen 1969)

The present observations are compatible with those of Robb (1963) who observed thrombo-embolism in the vascular beds of bowel, mesentery liver and lung in bled rabbits Thrombi of various types usually consisting of masses of red cells bound together with fibrin have been found in biopsies from various tissues (see Hardaway 1966) As the nutrition of the tissues must be seriously endangered by such microvascular thrombotization it is not surprising that microvascular infarctions can be found following hemorrhage also in skeletal muscle Several other data suggest a widespread trapping of blood cells following hemorrhage (Gibson *et al* 1957) Furthermore there is a progressive decrease in the level of coagulation factors (Hardaway and McKay 1963 Lewis and Blattberg 1964) as well as a dramatic shortening of the clotting time *in vitro* (Hardaway 1966) These data are compatible with disseminated intravascular coagulation

In this context it is interesting to note that Baeckstrom *et al* (1970) in fully heparinized cats obtained functional evidence for an extensive and rapidly occurring microplugging of the vascular bed of muscle after sudden severe blood loss This occurred even when the regional vascular bed studied was kept maximally dilated but it was not seen if the adrenal glands had been denervated prior to the blood loss Then however large i v infusions of adrenaline could again precipitate some microplugging Parallel to this a considerable red cell trapping occurred while blood viscosity *in vitro* fell largely in proportion to the hematocrit reduction The mechanism by which the intravascular obstruction was brought about was not quite clear but thrombocytes are likely to have played a key role probably associated with red cell aggregation Similar results were obtained by Bo and Hognestad (1971) studying the pulmonary circulation in the cat after hemorrhage They found an increase in the pulmonary vascular resistance after hemorrhage and could show that this was due to an aggregation of platelets in the pulmonary vessels

In the present experiments hemorrhage was always combined with some trauma inherent in the operative procedures. However, when aortic occlusion was performed instead of bleeding, microthrombus formation was never observed in the microvessels although perfusion pressure and the luminal adjustments of these vessels could be very similar to the same variables in the bled animals. In addition only moderate rouleau formation with a low cohesion between cells was then observed. This means that the micro thrombus formation cannot be due solely to a reduced flow rate in the microvessels. Changes in the properties of the blood and its cellular elements as well as in the blood endothelium relationship seem to be a prerequisite for the appearance of microthrombi.

These findings agree with those of Gelin (1956) who was unable to induce a stable aggregation in the vessels of the rabbit's cornea only by decreasing the regional flow rate. In cutaneous vessels of man Branemark (1971) found rouleau formation during reduction of blood flow, but even after 3 h of stagnation these rouleaux could be quickly broken apart by a re-established flow.

In bled animals the micro plugging in combination with the luminal adjustments resulted in an uneven capillary perfusion, the capillaries emerging from the distal parts of the transverse arterioles being the first to become occluded, with the flow restriction gradually spreading to more proximal capillaries. Finally a whole network could be obstructed or more often flow remained only in a few capillaries emerging from the more proximal part of the transverse arteriole.

The correspondingly increased diffusion distances must impose upon the tissue a decreased exchange between blood and cells and a state of functional shunting is established. These findings are in agreement with those of Baeckstrom *et al.* (1970) who found that in bled heparinized cats the filtration coefficient could remain markedly reduced even upon massive infusions of vasodilator drugs, strongly suggesting an extensive plugging of the capillaries. This has also been shown in dogs by Appelgren and Lewis (1972) with tissue clearance methods.

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Microvascular Dimensions and Blood Flow in Skeletal Muscle

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Abstract

ERIKSSON, E and R MYRHAGE *Microvascular dimensions and blood flow in skeletal muscle* Acta physiol scand 1972 86 211—222

In cats anesthetized with chloralose the lengths and diameters of the different vascular segments in the tenuissimus muscle were measured in the vital microscope The flow characteristics including red cell velocity were registered Fixed and stained muscle specimens were analyzed histologically as well as Indian ink perfused muscles treated according to the method of Spalteholz The average muscle fibre diameter was 44 μm 62 % of the muscle fibres were red (48 % B-fibres diameter 41 μm and 14 % C fibres diameter 26 μm) and the remaining 38 % were white (A fibres diameter 55 μm) The average muscle fibre was surrounded by 3 6 capillaries The capillaries were 1015±16 3 (S E) μm long and 5 3±0 0(3) (S E) μm in internal diameter The calculated capillary surface area was 0 9 m^2/100 cm^3 of muscle tissue Average red cell velocity in the capillaries was found to be 0 5 mm/s but there was a wide variation with time and between different capillaries Neither spontaneous changes in internal diameters of the microvessels nor a distinct precapillary sphincter activity could be observed Arterio-venous anastomoses within the muscle tissue were rare exceptions

The vascular arrangement in skeletal muscle muscle has been studied very carefully by Spalteholz (1888) Krogh (1919 1959) extended these studies and tried to make numerical calculations Since then a large number of investigators have tried to give a numerical picture of the vascular tree in different skeletal muscles A critical analysis of these studies is given by Hammersen (1968) By simple numerical calculations he showed that some of the figures presented earlier must be incorrect and also claimed that the vascular architecture is not as regular as Spalteholz and Krogh assumed

In vivo microscopic studies of the circulation in skeletal muscle were presented by Rous *et al* (1930) Subsequently about a dozen different methods have been used The best optical resolution has been obtained by Hyman and Paldino (1962) and Gray (1971) using the spinotrapezius muscle and by Baez (1969) and Smaje *et al* (1970) using the cremaster muscle in the rat Smaje *et al* were able to measure vascular dimensions and make numerical calculations on the basis of these dimensions They have also been able to record pressures in the microvessels

In the present experiments the tenuissimus muscle in the cat has been exposed for intravital microscopy according to the method described by Branemark and Eriksson (1972) This technique provides an optical resolution that admits analysis of the changes in the vascular diameters and in the rheological properties of the blood (Eriksson and Lisander 1972a) In order to obtain a good numerical picture of the vascular architecture in this muscle intravital microscopy has been combined with light microscopy of stained and unstained specimens and 3 D microscopy of microangiograms

Methods

26 cats of in average 2 5 kg were used They were anestetized with chloralose (50 mg/kg of b w) and were breathing spontaneously

According to the method described by Branemark and Eriksson (1972) the tenuissimus muscle in cat is exposed for intravital microscopy blood vessels and nervous supply being left intact. During the preparation and observation period the muscle is kept moistened by drips of Tyrode s solution of 35 C to which Dextran is added in a concentration giving neither shrinkage nor swelling of the muscle fascia The muscle is placed at the same horizontal level as the right atrium *i e* the venous pressure in the veins draining the tenuissimus muscle is about the same as the central venous pressure The hip joint of the cat is bent 90 and the knee joint 90 in order to standardize the tension in the muscle in the different cats

Objectives ×4 NA 0 14 ×10 NA 0 30 ×23 NA 0 55 ×55 NA 0 84 ×75 NA 1 00 are used for the intravital studies In order to minimize the influence of the light on the tissue a green filter with maximal transmission at 5500 Å is used The measurements of internal diameters and lengths of vascular segments are carried out with an optical micrometer Linear flow rates are estimated with a flying spot method (Brånemark and Jonsson 1963) Cinemato- and photomicrographic registrations are performed in most experiments The microscope is also connected to a closed circuit TV system with a videotape recorder These recordings are then used for calculations of blood cell velocity and plasma spacing (capillary hematocrit) The venous outflow from the hind limb musculature was in some experiments measured with an optical drop recorder in the femoral vein

In three animals the hindlegs were perfused with 3 % glutaraldehyde (buffered with 0 0/5 M sodium cacodylate to pH 7 2) This gives an almost instantaneous fixation of the vascular endothelium and a rapid diffusion into the other layers of the vessel and the surrounding tissue In seven other animals the muscle (exposed for vital microscopy) was fixed by drips of glutaraldehyde Before fixation the leg of the cat was always bent as described above in order to fix the muscle in the same state of stretch The fixed muscle biopsies were then cut in sections 1–? μm thick Some om them were analysed unstained some of them stained according to the van Gieson method With this treatment of the muscle the dimensional changes are less than 5 % (Branemark and Ekholm 1969) The number of muscle fibres in a cross section of the muscle and their diameters were measured in these specimens

In six cats the tenuissimus muscles were treated according to the method of Spalteholz (18?? see also Romeis 1948) The hindlegs are perfused with Indian ink (to which papaverine is added to induce maximal dilation) The muscles are then fixed in 5 % formaline solution After dehydration in ethanol the muscle is put into a mixture of 85 % Benzolbenzoate and 15 % Wintergreen oil B this procedure the muscle tissue becomes quite transparent and the vessels are distinctly delineated The whole vascular architecture can then be analysed in a 3 D microscope

In order to demonstrate the different types of red and white muscle fibres biopsies of 10 tenuissimus muscles were stained for adenosine triphosphatase as reported by Henneman and Olson (1965) The muscle biopsies are frozen in liquid nitrogen Cross-sections are obtained in a cryostate at −18 C Mounted fan-dried sections are fixed in Novikoff's calciumformol Mitochondrial ATP ase at pH = 7 2 is demonstrated by the lead method The medium also contains ATP magnesium sulphate and tri maleate buffer Dinitrophenol an activator of mitochondrial ATP ase is added to the medium in order to counteract the inhibition resulting from fixation The sections are incubated at +37 C and immersed in ammoniumsulfide and then mounted without dehydration Formation of lead phosphate is due to the existence of mitochondrial ATP ase Subsequent treatment with ammonium sulfide results in a dark brown precipitate of lead sulfide In accordance with their degrees of ATP ase activity and the size and distribution of the mitochondria the fibres are turning more or less dark brown On the

TABLE I

Type of muscle fibre	A	B	C	Mean value
Distribution of fibres ( )	38	48	14	
Muscle fibre diameter (μm)	55	41	26	44
Number of capillaries surrounding one muscle fibre	3 5	3 6	3 8	3 6

basis of the criteria of Henneman and Olson the tenuissimus muscle fibres are then classified as A B or C fibres The diameters of the different fibres are measured and the capillaries surrounding each type of fibre are counted

Results

The tenuissimus muscle is a thin and slender muscle running from the sacral and coccygeal bones down to the crural fascia The total number of muscle fibres in a cross section of the muscle is on an average 1375 The average fibre diameter is 44 0 μm The distribution of red and white fibres is shown in Table I In the cats used the muscle has a width of about 3—5 mm and a thickness in the central part of 0 3—0 6 mm In the borders it is not more than 0 05—0 1 mm thick

The feeding vessels to the muscle do not supply any other muscles and the vessels within the tenuissimus muscle do not anastomose with vessel from other muscles One *feeding artery and one draining vein enter the muscle together* (occasionally there are one artery and two veins running together) The average internal diameter of the feeding artery is 110 μm that of the vein being 165 μm The average distance between 2 such pairs of vessels is 25 mm These vessels are connected to the central artery and central vein which run parallel to the muscle fibres (Fig 1) Adjacent to them runs a nerve with a diameter of 50—100 μm The average internal diameter of the central artery is 72 μm whereas that of the central vein is 89 μm The transverse arterioles* and venules* branch off from the central vessels at angles of 45—90° They thus run transversely or diagonally to the muscle fibres (Fig 2) At the beginning the transverse *arteriole has a diameter of 22 μm and the transverse* venule 40 μm The average distance between two transverse arterioles is 650 μm and between two transverse venules 540 μm The distance between a transverse arteriole and the nearest transverse venule can vary from some 10 μm up to 1000 μm A transverse arteriole has on an average 11 branches (Fig 9 a Fig 4) Correspondingly the transverse venule has on an average 14 branches (Fig 9 b) The consecutive arteriolar branches then divide in an almost dichotomous pattern Unlike the transverse arterioles the transverse venules subdivide more like the branches of a fir On the arteriolar side most of the divisions are dichotomous (approximately 60 %) but since so many of them are non dichotomous it is not possible to divide

* For simplification vessels larger than 50 μm are called arteries and veins while those smaller than 50 μm are called arterioles and venules Rhodin (1967) presents a definition of the nomenclature of the microvessels based on the vascular morphology

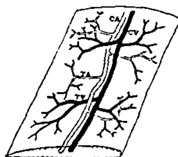


Fig 1

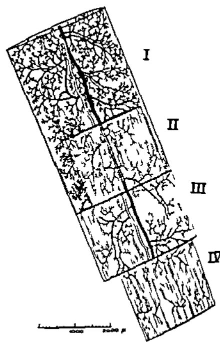


Fig 2

Fig 1 Schematic representation of the vascular arrangement in the tenuissimus muscle CA = central artery CV = central vein TA = transverse arteriole and TV = transverse venule

Fig 2 A detailed schematic representation of the vescular architecture of the tenuis mus muscle Arterial vessels—open venous essels—filled Sections of different depths are made into the muscle at II III and IV At I a projection of the pre and postcapillary vessels is hown The section at II shows the vessels above at III at the same and at IV under the le el of the central vessels

the *whole* vascular tree into different vascular generations The lengths and internal diameters of consecutive segments are shown in Fig 9 a b

After an intravascular distance of 1000—2000 μm from the central artery the mallest arterioles subdivide into capillaries (Fig 3) In this context a capillary is defined morphologically as beginning at the point of the last division of the arteriole (see Fig 3 and 5) It then runs parallel to the muscle fibres The end of the capillary will be where it joins another capillary to form a small venule or empties directly

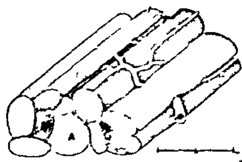


Fig 3 A graphic representation of a small arteriole subdividing into capillaries The capillaries are then running parallel to the muscle fibres

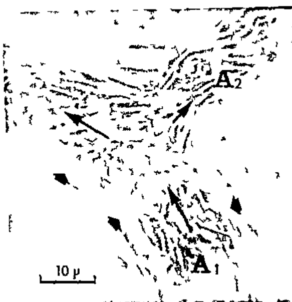


Fig 4



Fig 5

Fig 4 An arteriole (A1) of 20 µm giving off a branch (A) of 12 µm Note the wall n ar plasma la er in the larger essel indicated by thick arrows

Fig 5 A small arteriole (A) of 6 µm dividing into tw o capillaries (C) E is the nucleus of an endothel al cell

into a significantly larger venule at right angles The average capillary in the tenuis simus muscle has a length of 1015/±163 (S E)/µm and a diameter in the middle of 5 3/±1 1 (S D) or ±0 0(3) (S E)/µm (see Fig 10) A given capillary is con nected to a neighbouring capillary by an anastomose at every 200 µm on an average The ana tomoses are somewhat closer in the venular part of the capillary In a given area of the muscle the number of capillaries is about 40 % larger at the venular end of the capillaries than at the arteriolar end At the arteriolar end the capillary has a diameter of 4 7/±0 9 (S D) or ±0 0(3) (S E)/µm compared to 5 9/±0 8 (S D) or ±0 0(2) (S E)/µm at the venular end The total number of capillaries in a cross section of the tenuissimus muscle is 1310 (ATP ase stined muscles) This gives a capillary/fibre ratio of 0 95 When counted in the ital microscope or in specimens prepared according to the method of Spalteholz the figure obtained is about 1250 An A fibre (diameter 55 µm) is on an average surrounded by 3 5 capillaries a B-fibre (diameter 46 µm) by 3 6 and a C fibre (diameter 26 µm) by 3 8 capillaries (see Table I)

In the feeding and the central vessels the direction of flow is always the same This is also the case in the arterioles and most of the venules but it can be reversed in the capillaries and the small venules Moreover the linear flow rate in the capillaries was found to vary from zero up to 1 5 mm/s (average flow velocity in a capillary 0 5 mm/s) The flow velocity in the intercapillary anastomoses is mostly

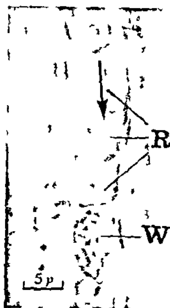


Fig 6



Fig 7

Fig 6 A capillary of 6 µm in the tenuissimus muscle W = white cell and R = red cells The white cell is deformed in the vessel and causes a reduction of the flow velocity in the capillary

Fig 7 Cross section of the tenuissimus muscle fixed by perfusion with glutaraldehyde Note that there are about as many capillaries around the small (red) fibre as around the large (white) one c = capillaries and a = intercapillary anastomose

flow indicating a small hydrostatic pressure gradient between 2 neighbouring capillaries The flow in these anastomoses is very often reversed During short periods there is only plasma flow in a capillary with the occasional passage of a platelet

In a given area of the muscle most of the capillaries come from one and the same arteriole and discharge into the same venule This means that the capillary flow direction in such an area is the same in all the capillaries This arrangement excludes fairly efficiently a counter-current effect in this type of muscle

With the method used it has not been possible to observe any spontaneous changes in diameter in the vessels in the tenuissimus muscle under steady state resting conditions All the capillaries in the muscle are always open to flow even if flow velocity can vary drastically in different capillaries Sometimes the flow in a capillary seems to slow down and even stop due to the passage of or blockage by a white cell (Fig 6

It has not been possible to observe any distinct pre-capillary sphincter activity*

Arterio-venous shunt like vessels are very rare exceptions within the tenuissimus muscle In about one muscle out of twenty we have observed one such vessel with a contractile wall being about 15 µm in diameter and 500 µm in length Within the

* A pre-capillary sphincter is defined as the last smooth muscle cell along any branch of a terminal arteriole (Wiedeman 1963 Due to its activity the lumen of the vessel can be open or closed (Chambers and Zweifach 1944



Fig 8 Cross section of the tenuissimus muscle fixed in liquid nitrogen and stained with mitochondrial ATP ase The white A and the red B- and C-fibres can be recognized (see Henneman and Olson) The space between the muscle fibres is much larger here than in Fig 7

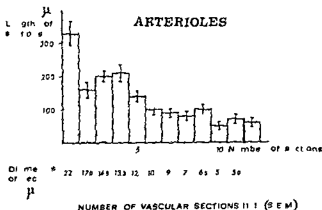
muscle fascia on the other hand there are one or two shunt like vessels in every muscle observed connecting a transverse arteriole and venule These vessel are about 12 μm in diameter and 1000 μm in length and they contract in i a injection of norepinephrine When the cat is fat having fat cell scattered in the muscle fascia these vessels seem to arborize and nourish the fat cells

Discussion

A morphological study of the vascular bed in skeletal muscle has its limitations *e g* due to the artifacts created by the histological preparation In order to improve the possibilities of analysis we have combined morphological methods with intravital microscopy We have also chosen to concentrate our studies to the tenuissimus muscle of the cat so as to arrive at as good a description of the vasculature in this muscle as possible

A question of importance is how representative the tenuissimus muscle is of skeletal muscle in general When taking the muscle fibre diameter and the distribution of red and white muscle fibres into consideration the circulatory findings in the tenuissimus muscle may probably be valid for other muscles in the cat as well This however remains to be shown

Another question is how representative of a resting state this muscle is when treated according to the present method The dissection is always performed very carefully We also try not to put any tension on the muscle During the preparation and observation period the muscle is kept moistened by drips of Tyrode s olution of 35° C to which Dextran is added At administration of vasodilator drugs like acetylcholine or papaverine the arterioles can dilate up to double the resting diameter (Eriksson and Lisander 1972 a) Sympathetic stimulation may decrease the arteriolar diameters down to 1/5—1/10 of the resting values depending on what vessels that are observed (Eriksson and Lisander 1972 a) During prolonged experiments the muscle has been exposed in the microscope for 8 h without signs of tissue injury or impaired blood flow When the total blood flow from the isolated left hindleg is measured with a drop recorder (when the tenuissimus muscle is exposed



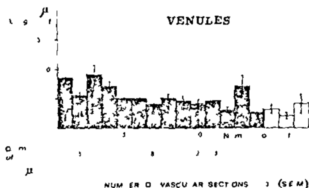


Fig 9 The numbers lengths and diameters of the different sections of the transverse arteriole (9 a) and transverse venule (9 b) are shown in the diagrams The segment of transverse vessel between 2 branches is defined as a section The presented diameters are internal diameters measured 50 μm from the proximal end of the section or in the middle of it if the section is shorter than 100 μm Standard error of the mean (S E) is given for the numbers and lengths For the diameters the S E is of the order of ±0 5

in the microscope) the obtained values are 3—6 ml/min and 100 g muscle tissue which is fairly normal for resting blood flow in skeletal muscle (see Mellander and Johansson 1968)

The vascular bed in the tenuissimus muscle is only connected to its neighbourhood by its feeding vessels there being no anastomoses to other muscles The fact that the muscle has a number of feeding vessels instead of one single artery and vein entails the disadvantage that it is technically almost impossible to isolate and measure its total venous outflow According to the method described by Johnson (1971) the sartorius muscle of the cat can be isolated at the same time as the microcirculation is observed This method has on the other hand the disadvantage of giving rise to a greater preparation trauma

The tenuissimus muscle is very thin and so does not provide a three-dimensional picture of the architecture of the main vessels within a muscle A constant finding is the central vessels running parallel to the muscle fibres Preliminary studies on other muscles (Eriksson and Myrhage to be published) shows that the arrangement with central vessels and transverse or diagonal branches also holds for thicker muscles In thicker muscles the transverse vessels branch off like the branches of a fir A pair of central vessels thus s[illegible] a cylinder of muscle tissue

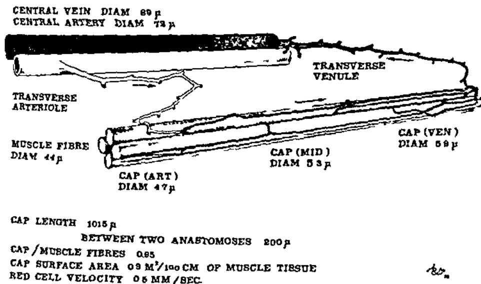


Fig 10 Summarizing illustration of the vascular dimensions in the tenuissimus muscle

The vascular arrangement in the tenuissimus muscle is similar to the general picture given for a skeletal muscle by Spalteholz (1888). There are however some dissimilarities The arterioles can sometimes run more or less diagonally to the muscle fibres and not always transversely as Spalteholz found He also described the capillaries as running from the arteriole to the nearest venule This was not always so in the present examinations of the tenuissimus muscle

A V anastomoses within the muscle are practically never seen in the tenuissimus muscle In the connective tissue surrounding the muscle there are however occasional vessels about 12 μm wide and 1000 μm long running directly from the transverse arteriole to the transverse venule These vessels do not anastomose with the capillaries within the muscle When there are fat cells close to these vessels capillaries branch off from them nourishing these cells These data do not agree with the results of Krogh (1959) and Algire (1954) who described A V anastomoses in the muscles they examined

In the tenuissimus muscle it has neither been possible to prove the existence of metarterioles (Zweifach and Metz 1955) nor to find the macro capillaries of Saunders *et al* (1957) The present findings on these points agree well with the results of Hammersen (1968)

Distinct pre capillary sphincters have not been observed in this muscle However under steady state resting conditions the linear flow rates in the capillaries vary to an enormous extent but this could not be ascribed to any obvious changes in the diameters of the vessel We have not been able to show any spontaneous changes in the internal diameters of arterioles venules or capillaries under steady state resting conditions (which include a constant perfusion pressure) Sometimes the flow rate

in a capillary can be reduced due to the passage of a leucocyte (Fig 6) In two neighbouring capillaries the linear flow rates are often very different and seem to change markedly without any rhythmicity We have observed that the fewer red cells there are in a capillary the higher the flow velocity On the other hand the more cells there are in a capillary, the lower is the linear flow rate compared to that in other capillaries This can be explained by the fact that the capillaries are about 5 3 μm in internal diameter while the red blood cells in the cat are about 6 μm. When a red cell passes through a capillary it will somewhat increase the resistance in this capillary compared to the situation when only plasma passes through

In another study (Eriksson and Lisander 1972 a) evidence is presented indicating that arterioles of 10—30 μm can act as a kind of pre capillary sphincters (see Fig 4) During prolonged sympathetic constrictor fibre stimulation these arterioles will first constrict but then they will dilate intermittently and for short periods These rhythmic changes in arteriolar diameter could have the same influence on capillary flow as pre capillary sphincters On the other hand flow differences between closely adjacent capillaries cannot be explained by such a mechanism because one arteriole of this diameter supplies some hundred capillaries, and diameter changes in arterioles smaller than 10 μm have not been observed in this muscle These data do not agree with the findings of Zweifach and Metz (1955) who described pre capillary sphincters in the spinotrapezius muscle of the rat The distribution of the blood through the capillaries remains even in the tenuissimus muscle even at topical administration to such precapillary arterioles of different doses of pinephrine a treatment which makes arterioles of 10—30 μm constrict markedly

* When the perfusion pressure decreases Algire (1954) has reported that capillaries and veins collapse When in the present preparation the perfusion pressure was lowered by means of an aortic clamp the venules begin to collapse when the femoral artery pressure falls below 35 mm Hg (see Eriksson and Lisander 1972 b) However we have never seen capillaries collapse even when the femoral artery pressure is as low as 10 or 15 mm Hg

The average internal diameter of the capillaries was found to be 5 3 μm in the middle of the capillary (Fig 10) It is narrower at the arteriolar end (4 7 μm) and wider at the venular end (5 9 μm) These figures represent average values of 1775 capillaries measured *in vivo* It should be pointed out that there is a wide variation in capillary diameter in the muscle It is possible to find capillaries with diameters (at the middle) ranging from 2 5 to 8 μm The calculated standard deviation on this parameter was found to be 1 1 μm The present findings are in good agreement with those of Smaje *et al* (1970) who found a capillary diameter of 5 5 μm in the arteriolar end and 6 1 μm in the venular end in the cremaster muscle of the rat

In the tenuissimus muscle the fibre diameter is on an average 44 μm the number of capillaries/muscle fibres being 0 95 These figures will give 0 8 m² capillary surface area/100 cm³ of muscle tissue We have then calculated with a ratio of muscle fibre volume to connective tissue + vessel volume of 8 2 If we then add the surface

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Coding of Amplitude and Frequency Modulated Sounds in the Cochlear Nucleus of the Rat

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Abstract

MØLLER A R. *Coding of amplitude and frequency modulated sounds in the cochlear nucleus of the rat* Acta physiol scand 1972 86 223—238

The responses of single units in the cochlear nucleus of the rat to sinusoidally amplitude and frequency modulated tones and amplitude modulated broadband noise were studied The distribution of discharges within a cycle of modulation was determined from cycle histograms locked to the modulation wave In response to amplitude modulated tones and broadband noise all units investigated showed a peak in the degree of modulation of the spike frequency within the modulation frequency range from 50 to 200 Hz. In many units the relationship between the degree of modulation of the stimulus sound and of the modulation of the resulting spike train was almost unchanged over a wide range of sound intensities In other units enhancement of modulation within a certain range of modulation frequency became more pronounced when the sound intensity was increased This was mainly due to a suppression of modulation at lower modulation frequencies The shape of the histograms was nearly sinusoidal even at modulation depths which resulted in nearly 100 modulation of the neural discharge frequency The amount of modulation of the discharge frequency in response to frequency modulated tones was dependent on the frequency of the tone in relation to the CF of the unit

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The intensity of natural sounds usually varies with time at various rates Many sounds have a more or less rapidly changing spectral composition The wellknown static description of the response areas of single units in the ascending auditory pathways with regard to frequency and intensity reflects only the static response and does not contain information about how the responses are related to the dynamic parameters of the sound Only a few studies have reported on the influence on firing in primary Eighth Nerve fibers and cochlear nucleus units in response to sounds where the parameters varied with time

In the present study the responses of single units in the rat cochlear nucleus have been recorded in response to sinusoidally amplitude modulated pure tones and broad band noise and to sinusoidally frequency modulated tones [1] The resulting modula

[1] The spectrum of a sinusoidally amplitude modulated tone consists of 3 discrete components the carrier (f) and 2 side frequencies the frequency of which is equal to the carrier frequency plus or minus the modulation frequency (f + f and f − f_m) The degree to which a carrier is amplitude modulated is specified by the ratio between the envelope and the steady

tion of the neural discharge frequency has been quantitatively determined from cycle histograms locked to the modulation waveform

Sinusoidally amplitude modulated stimuli have been used in quantitative studies of the dynamic properties of the sensory neural transmission in vision (see *e g* Cleland and Enroth Cugell 1966) proprioception (see *e g* Matthews and Stein 1969) and between neurons (see *e g* Watanable 1962 Terzuolo and Bayley 1968)

Generally it has been found that the gain functions which relate the frequency modulation of the recorded discharges to the modulation of the stimuli resemble those of a lowpass filter In some situations such gain functions have been shown to have a more or less pronounced peak near the cut-off frequency representing a tuning to a particular modulation frequency (Ratliff *et al* 1967 Ratliff *et al* 1969)

In the auditory system such investigations have been sparse Using chopped tones and noise Glattke (1969) found that the firing of many units was evoked in more or less perfect synchrony with modulation up to modulation rate of 500 Hz and occasionally to 800 Hz He judged the degree of synchrony from dot displays of the neural discharges presented on successive sweeps locked to multiples of the modulation frequency

This suggested that a quantitative investigation should be carried out concerning the dynamic properties of the coding of amplitude and frequency modulated tones.

Methods

The sound stimulating system surgical procedure and recording technique was the same as described earlier (Moller 1971 The stimulus patterns used in the present study consisted of sinusoidally amplitude modulated pure tones and broad band noise and sinusoidally frequency modulated pure tones In the case of the broad band noise the electrical input to the sound source was uniform in the frequency range of interest Thus the sound spectrum at the eardrum was identical to the frequency characteristic of the sound generating system (see Møller 1971) The modulation frequency was usually varied in the range from 1 to 1000 Hz In the case of amplitude and frequency modulated tones the frequency of the tone (carrier frequency) was kept constant either equal to the CF of the unit or in some cases slightl higher or l wer

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arrier amplitude as is illustrated in Fig 1 This ratio is called the modulation index or "depth of modulation Expressed in percentage of the steady carrier amplitude it is usually called "percentage of modulation When the degree of modulation is increased more and more of the energy is redistributed from the carrier to these side frequencies In a 100 modulated tone the carrier represents 50 and each of the two side frequencies 25 of the total energy In the experiments described in this paper the modulation was not synchroneou with the carrier The carri r frequenc thus had a different phase relation to the envelope frequency in subsequent period of the modulation waveform When such a modulated signal is viewed on an oscilloscope the sweep of which is synchronized to the envelope frequency a blurred image is seen

The spectrum of a frequency modulated signal i more complicated than that of amplitude modulation and it contains an infinite series of side frequencies At low modulation index however the amplitudes of side frequencies other than the first pair ($f + f_m$ and $f - f_m$) are small The amplitude spectrum of such a FM signal thus resembles that of an AM signal The phase angle of the spectrum however is different the side frequencies of the FM signal be re shifted 90 compared t that of an AM signal see *e g* Cherry 194)

When the modulation index is increased the energy of the higher order modulation components ($f + 2f_m$ f $3f_m$ $f - 2f_m$ $f - 3f_m$) becomes significant and the influence of these components in r es as the amount f modulation is increased At high amounts of modulation a widespr d series pe tral compon nt is generated

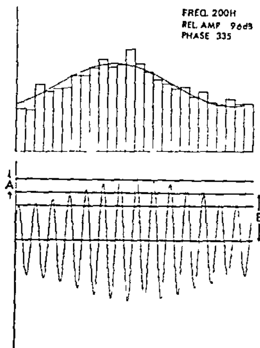


Fig 1 A cycle histogram of the activity recorded from a neuron in response to an amplitude modulated sound like that illustrated below The relative amplitude of the modulation of the cycle histogram is given i dB re 100 % modulation (−9 6 dB corresponds to 33 % modulation)

but always within the response area of the unit The amplitude modulation of the sound was produced by means of a 4 quadrant analogue multiplier (Motorola MC 1595L) The frequency modulation of the sounds was produced by feeding a sinusoidal signal into the frequency control input of a function generator (Wavetek Model 112)

Usually samples of 30 s duration were presented at each modulation frequency Nerve impulses were recorded on magnetic tape together with synchronization pulses which marked the beginning of a modulation period The recorded nerve discharges were analysed (Intertechnique DIDAC 800) to produce cycle histograms with respect to the modulation frequency An example of a cycle histogram is seen in Fig 1 In order to select an optional fraction of the total number of channels of the analyzer it was modified so that each synchronization pulse could return the sweep of the analyzer to its initial position immediately before initiating a new sweep The digital content in each of the analysis channels was punched on paper tape in binary form The 8 most significant bits were punched on 8 channel paper tape by a paper tape punch (Facit 7040) The paper tape was later read into a general purpose digital computer (IBM 360/75) which was programmed to fit one or more sinusoidal waves to the histograms This was done by computing the Fourier transforms of the histogram and then synthesizing a wave on the basis of the computed Fourier coefficients The amplitude and phase angles of these waves were printed and used as a measure of the degree of modulation of the neural discharge frequency Such a wave fits the original curve according to the least mean square error criterion (see e g Sokolnikoff and Redheffer 1958) The overall dynamic range of the system was slightly more than 40 dB

Since this study is based on responses to continuous sound stimulation particular attention had to be paid to the effect of adaptation The mean firing rate was determined during the entire experiment in cases it changed more than about 20 % during the recording the data were excluded Furthermore each recording sequence was begun with modulation at 100 Hz. The results were compared with those of a check stimulation with 100 Hz modulation at the end of the recording sequence The difference between these two recordings was usually less than 1 dB if it was more than 2 dB the data were excluded

In some cases stimulation consisted of 5 s of sound alternating with 5 s of silence The results obtained with this type of stimulation did not differ significantly from those where the sound was continuous

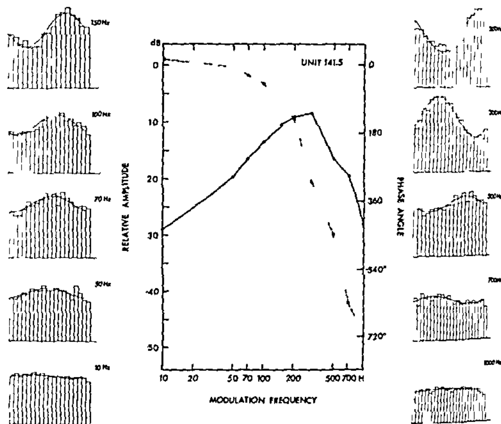


Fig. 2. Cycle histograms of the activity of a unit in the cochlear nucleus in response to amplitude modulated sounds. The smooth curves are sinewaves that fit the histograms according to the least mean square criterion. The modulation frequency is indicated by legend numbers. The middle graph is a Bode plot or gain function which shows the amplitudes (in dB: solid lines) and phase angles (dashed lines) of the sinewaves that fit the different histograms plotted as a function of modulation frequency. The ordinate is the amount of modulation relative to 100 % (0 dB). The modulation of the stimulus tone was 20 % and its frequency was equal to the CF of the unit (30.0 kHz). The stimulus intensity was 60 dB SPL and the threshold of the unit was 45 dB SPL.

Results

Amplitude modulation

The results of the present study were based on recordings from 53 units in 16 animals. Only units which could be maintained for more than 30 min were included. Responses were obtained from many units for as long as 2 h and in certain cases for more than 4 h. Units were encountered from the entire cochlear nucleus complex.

Fig. 1 shows an oscillogram of the utilized amplitude modulated sound together with a histogram of the neural discharges of a unit in the cochlear nucleus of a rat in response to the amplitude modulated tone. The smooth curve superimposed on the histogram is a single sine wave matched to fit the histogram according to the least mean square error criterium. The figures in the upper corner of this diagram give

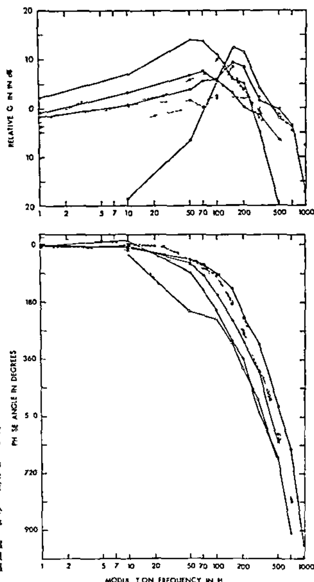


Fig 3 Gain functions showing the ratio between the modulation of the discharges and the amplitude modulation of the stimulus sound together with the phase angle between the modulation of the discharges and the sound Results from 8 typical units are shown the frequency of the tones was equal to the CF of the units The sound intensity was in all cases about 20 dB above threshold and the CFs of the units ranged from 9.0 Hz to 30 kHz

the relative amplitude and the phase angle of the matched sine wave The degree of modulation of the amplitude modulated tone is the ratio between A and B It is usually expressed in percent

The cycle histograms which were locked to the modulation wave show the distribution of discharges within one cycle of the modulation wave The variations of the discharge frequency during the modulation cycle are reflected in the cycle histogram which thus is a measure of the modulation of the density of neural discharges

It is seen that a sinusoidally amplitude modulated tone gives rise to a discharge

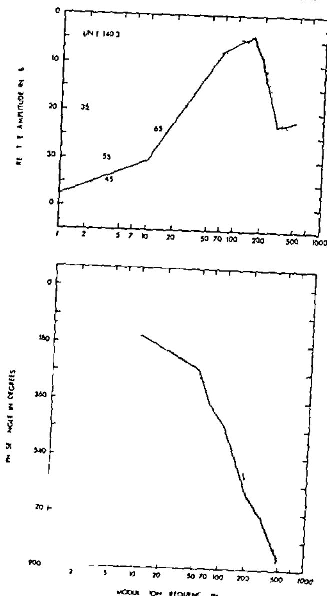


Fig 4 The relative amplitude of the modulation of the discharges of a unit to amplitude modulated tones at CF for different sound intensities The modulation of the tones was 20 % Numbers on the individual curves give the sound pressure level (SPL) in dB The CF of the unit was 15.? kHz and its threshold was about 20 dB SPL The firing rate of the unit had its maximal value at 40 dB SPL Above that level the firing rate decreased again

frequency which in a first approximation varies sinusoidally as a function of time The amplitude and phase angle of the sinusoidal wave which fits the cycle histogram varies as a function of the modulation frequency in a characteristic way This can be seen from Fig 2 which shows the relative amplitude (in dB) and phase angle (in degrees) of the sine waves which fit the histograms in the range of modulation frequency from 10 to 1000 Hz together with the cycle histograms of the discharges at the different modulation frequencies The stimulus was an amplitude modulated pure tone at CF (30.0 kHz) the modulation was 20 % and the intensity of the tone was 60 dB SPL (re 0.0002 μb) The threshold of the unit was 15 dB SPL In the

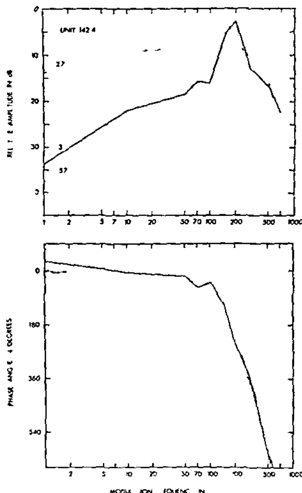


Fig 5 Gain functions similar to those in Fig 4 Numbers on the curves give the sound pressure level in dB SPL The carrier frequency was equal to the CF of the unit (21 9 kHz) and its threshold was about 17 dB SPL The modulation of the tone was 30

following figures the relative amplitude of the modulation (i e ratio in dB between the peak amplitude of the sinewave that matches the histogram and the mean height of the cycle histogram) is plotted as a function of frequency The value 0 dB thus corresponds to 100 % modulation The phase angle (in degrees) between the modulation wave and the sinewave matched to fit the cycle histogram is also plotted as a function of frequency Such diagrams are often called gain functions or Bode plots and they represent the frequency domain transfer function of the system with regard to modulation

It is seen from Fig 2 that there is a pronounced enhancement of the modulation wave in the neural discharge pattern at or around a modulation frequency of 300 Hz At this frequency the modulation of 20 % i e of about ± 1 6 dB gives rise to nearly 40 % modulation of the density of the recorded neural spike train

Other units however have a less pronounced peak in their gain function Fig 3

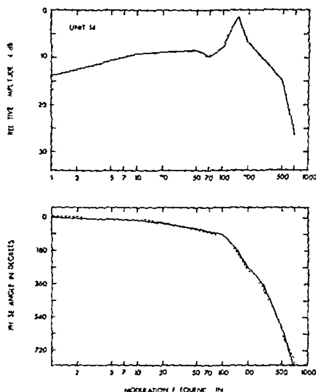


Fig 7 Gain functions based on the responses to amplitude modulated tones at CF (solid lines) and broad band noise (dashed lines) The modulation was 30 % for both tones and noise The CF of the unit was 21 9 kHz and the intensity of the tone was 27 dB SPL The noise had a spectrum level of − 10 dB SPL (energy in a 1 Hz band) The threshold of the unit at CF was 17 dB SPL

were influenced very little by the change in sound intensity *Fig* 5 shows an example of such a response pattern In that unit the sound intensity was varied from 27 dB to 57 dB SPL (corresponding to a level of 10 to 40 dB above threshold) The modulation was 30 %

In certain units the shape of the gain functions obtained from responses to amplitude modulated sounds with different carrier frequency were similar as long as the carrier frequency was within the response area of the unit In other units the responses to amplitude modulated sounds change when the carrier frequency is shifted from the CF of the unit to a slightly higher or a lower frequency When the carrier frequency is increased above CF low modulation frequencies are usually not reproduced as well as when the carrier frequency is equal to the CF or lower This is illustrated in Fig 6a which shows the gain functions of the response of a unit to amplitude modulated tones with the carrier frequency (a) equal to the CF of the unit (15 200 Hz) (b) higher than the CF (16 200 Hz) and (c) lower than CF (13 900 Hz) There is an apparent change in the shape of the curves showing the relative amplitude of the modulation The phase angles are usually much less dependent on the frequency of the stimulus tone except that there may be a shift of 180 degrees, indicating that an increase in sound intensity at low modulation frequencies results in a decrease in firing frequency (a phase angle of 0 degree indicates that an increase in sound level causes an increase in firing rate cf Fig 1)

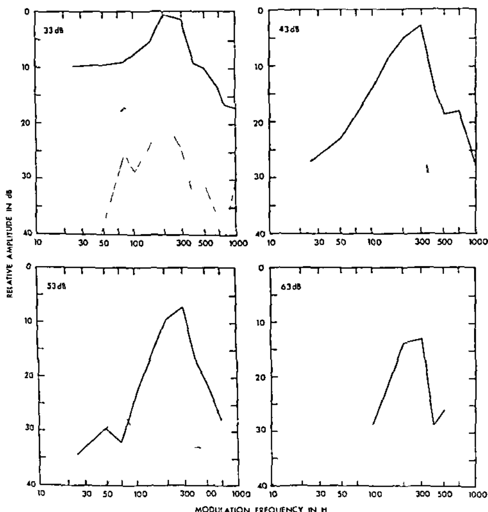


Fig 8 Relative amplitude of the fundamental frequency (solid line) of the second (dashed line) and of third harmonic (dotted line) wa e of the modulation of the discharges in response to amplitude modulated tones at four different intensities (given in each graph in dB SPL) The modulation of the stimulus tone was 15 % and the threshold of the unit at CF (?3 150 Hz) was 20 dB SPL

Similar to sinusoidally amplitude modulated tones the modulation wave of sinusoidally amplitude modulated broad band noise was also preserved in the recorded discharge pattern of cochlear nucleus units This is illustrated in Fig 7 which shows gain functions based on responses to amplitude modulated tones at CF (solid curve) and amplitude modulated noise (dashed line) of the same unit The modulation was 30 % for both tones and noise stimuli In some units however the amount of modulation of the di charge frequency has been found to be lightly

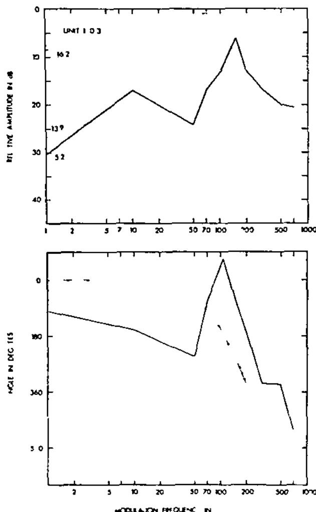


Fig 9 Gain functions of a unit in response to frequency modulated tones at CF (solid line) above its CF (dashed line) and below its CF (dotted line) The CF was 15 ? kHz and the frequencies used are indicated b legend numbers At 15 ? kHz the range of the modulation was ±300 Hz at 13 9 kHz±100 Hz and at 16 ? kHz it was±60 Hz This unit is the ame as that shown in Fig 6

less for noise as compared to tones The phase shift as a function of modulation frequency was however almost identical for amplitude modulated tones and noise

The distorsion of the shape of the cycle histograms was found to be rather small even in cases where the sound stimulation resulted in almost 100 % modulation of the neural discharge frequency The distorsion at various modulation frequencies was almost a constant percentage of the amount of the fundamental frequency of the modulation of the discharges When the sound intensity was increased the relative magnitude of the distorsion seemed to decrease This is illustrated in Fig 8 which shows the amplitude of the second and third harmonic together with the amplitude of the fundamental frequency of the modulation at four different sound intensities

In most of the experiments a modulation of 10 % was used In some experiments a higher degree of modulation was also used (e g 20 and 30 %) The resulting gain functions showed for the most part a parallel shift with a value equal to the increase

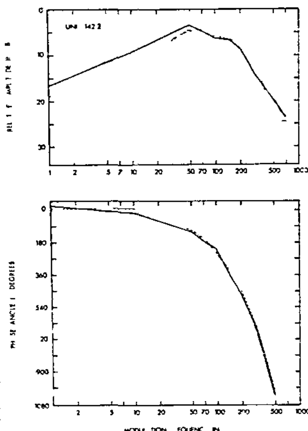


Fig 10 Gain functions based on the responses to amplitude modulated tones at CF (solid lines) frequency modulated tones at CF (dotted lines) and amplitude-modulated noise (dashed lines) The CF of the unit was 1500 Hz The intensity of the stimulus tones had a spectrum level of 12 dB was 40 dB SPL and the noise SPL (pr 1 Hz band) The amplitude modulation was 10 % and the range of frequency modulation was ±45 Hz

in modulation of the sound provided that the modulation of the spike frequency was below 100 % Thus these results show that there is a fairly linear relationship between the degree of amplitude modulation of a tone and the degree of modulation of the spike frequency of a single unit in the cochlear nucleus even at nearly 100 % modulation of the discharge frequency

Frequency modulation

The response to sinusoidally frequency modulated tones was studied in the same way as the response to amplitude modulated sounds It was assumed that a frequency modulated sound gives rise to a variation in the excitation in accordance with the shape of the response area of the unit The degree of modulation of the discharge pattern would then depend on the slope of the response area in the vicinity of the carrier frequency Fig 9 shows gain functions based on responses to frequency modulated tones of a typical unit when the carrier frequency was equal to the CF of the unit (15 2 kHz solid line) below its CF (13 9 kHz dotted line) and above its CF (16 2 kHz dashed line) As expected the modulation of the discharge frequency was more influenced by the frequency of the carrier than for amplitude

modulated sounds The modulation of the firing frequency was greater when the carrier frequency of the frequency modulated tone was near the steep flank of the response area of the unit than when the tone was located near the less steep low frequency flank The steepness of the high frequency flank was about 6 Hz/dB while the low frequency flank had a steepness of 100 Hz/dB If the response area is symmetrical around the CF the response to a frequency modulated tone at CF can be expected to give rise to a cycle histogram that resembles a double rectified pattern of the modulation waveform Above the CF of the unit an increase in tone frequency gives rise to a decrease in excitation while below it gives rise to an increase in excitation This results in a 180° phase shift between the response obtained at frequencies lower than CF compared to the response obtained at frequencies above CF In Fig 9 180 degrees were subtracted from the phase values that correspond to the case where the carrier frequency was higher than the unit s CF (16 2 kHz) in order to facilitate the comparison of phase angles In many units there was a tendency for the frequency modulation of a tone to be reproduced better at low modulation frequencies as compared with the modulation of amplitude modulated tones The phase relations are however usually nearly identical to that of amplitude modulation except in the case where the carrier frequency is equal to the CF of the unit The unit the responses of which are shown in Fig 9 is the same as that depicted in Fig 6a for amplitude modulated tones The sound intensities and frequencies are the same in these two figures The tuning curve of the unit is shown in Fig 6b A comparison between the two figures illustrates the difference in response pattern to and frequency modulated tones which is typical for many units In other units however the shape of the gain functions obtained from the responses to amplitude and frequency modulated tones are very similar even when the carrier frequence is close to the CF of the unit An example of such a unit is seen in Fig 10 which shows gain functions obtained with amplitude modulated tones (solid line) frequency modulated tones (dotted line) and amplitude modulated broad band noise (dashed line) The frequency of the stimulus tones was close to the CF of the unit (1 5 kHz) In this unit there was an almost identical response pattern to all three types of modulation

Discussion

The results of the present study show that small changes in the intensity of a tone or noise or in the frequency of a tone are enhanced in the discharge pattern of cochlear nucleus units in a certain range of rates of change in amplitude or frequency In the range of modulation frequencies where maximum enhancement occurs the amount of modulation of the neural spike frequency is usually greater than the amount of amplitude change of the stimulus tones and noise In a certain range of modulation frequency an amplitude modulation of 10 % (i e about ±0 8 dB) can result in more than 50 % modulation of the discharge frequency whereas the same degree of amplitude modulation at a low modulation frequency in the same unit may give rise to only approximately one percent change in firing rate

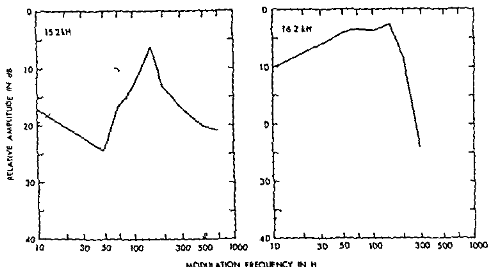


Fig 11 Gain functions based on the responses to frequency modulated tones at CF (left graph) and above CF (right graph) The CF of the unit was 15 2 kHz and the modulation was ±300 Hz and ±40 Hz respectively The sound intensity was 35 dB and 55 dB SPL respectively The relative amplitude of the fundamental (solid line) and that of the second harmonic (dashed line) wave of the modulation of the discharge frequency are shown as a function of modulation frequency

Thus enhancing of amplitude changes occurs within a certain range of rates and suppressing occurs at lower or higher rates The transfer functions (gain functions) relating modulation frequency of the stimulus to the frequency modulation of the neural discharges have a more or less pronounced peak at a certain modulation frequency The units can thus be regarded to function like a resonant filter which is tuned to a certain modulation frequency At that frequency very small changes in amplitude or frequency of a pure tone or in the amplitude of a broad band noise are clearly reproduced in the temporal pattern of the neural discharges It seems rather plausible that this enhancement of amplitude or frequency change in a certain range of modulation frequencies occurs in the neural excitation process in the cochlear nucleus

Sinusoidal modulation is reproduced with very little distorsion which suggests that even nonsinusoidal amplitude or frequency changes may be reproduced with great fidelity in the temporal pattern of the neural discharges The fact that the amount of distorsion (second and third harmonics) is an almost constant fraction of the fundamental component of the modulation in the discharge frequency points to an interpretation that the main source of distorsion is located after the filter which determines the response to the modulation If the distorsion had been generated at an earlier stage in the system the harmonic components would be enhanced when their frequencies coincided with the tuning of the neural filter The second harmonic would thus have been enhanced when the modulation frequency was equal to half the modulation frequency where the fundamental shows its highest value This was *not seen in responses to* any of the amplitude modulated

modulated sounds The modulation of the firing frequency was greater when the carrier frequency of the frequency modulated tone was near the steep flank of the response area of the unit than when the tone was located near the less steep low frequency flank The steepness of the high frequency flank was about 6 Hz/dB while the low frequency flank had a steepness of 100 Hz/dB If the response area is symmetrical around the CF the response to a frequency modulated tone at CF can be expected to give rise to a cycle histogram that resembles a double rectified pattern of the modulation waveform Above the CF of the unit an increase in tone frequency gives rise to a decrease in excitation while below it gives rise to an increase in excitation This results in a 180° phase shift between the response obtained at frequencies lower than CF compared to the response obtained at frequencies above CF In Fig 9 180 degrees were subtracted from the phase values that correspond to the case where the carrier frequency was higher than the unit s CF (16 2 kHz) in order to facilitate the comparison of phase angles In many units there was a tendency for the frequency modulation of a tone to be reproduced better at low modulation frequencies as compared with the modulation of amplitude modulated tones The phase relations are however usually nearly identical to that of amplitude modulation except in the case where the carrier frequency is equal to the CF of the unit The unit the responses of which are shown in Fig 9 is the same as that depicted in Fig 6a for amplitude modulated tones The sound intensities and frequencies are the same in these two figures The tuning curve of the unit is shown in Fig 6b A comparison between the two figures illustrates the difference in response pattern to amplitude and frequency modulated tones which is typical for many units In other units however the shape of the gain functions obtained from the responses to amplitude and frequency modulated tones are very similar even when the carrier frequence is close to the CF of the unit An example of such a unit is seen in Fig 10 which shows gain functions obtained with amplitude modulated tones (solid line) frequency modulated tones (dotted line) and amplitude modulated broad band noise (dashed line) The frequency of the stimulus tones was close to the CF of the unit (1 5 kHz) In this unit there was an almost identical response pattern to all three types of modulation

Discussion

The results of the present study show that small changes in the intensity of a tone or noise or in the frequency of a tone are enhanced in the discharge pattern of cochlear nucleus units in a certain range of rates of change in amplitude or frequency In the range of modulation frequencies where maximum enhancement occurs the amount of modulation of the neural spike frequency is usually greater than the amount of amplitude change of the stimulus tones and noise In a certain range of modulation frequency an amplitude modulation of 10 % (i e about ±0 8 dB) can result in more than 50 % modulation of the discharge frequency whereas the same degree of amplitude modulation at a low modulation frequency in the same unit may give rise to only approximately one percent change in firing rate

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From the Department of Anatomy Eye Research Laboratory University of Helsinki Finland

The Action of Physostigmine, Morphine, Cyclopentolate and Homatropine on the Secretion and Outflow of Aqueous Humour in the Rabbit Eye

By

RISTO UUSITALO

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Abstract

UUSITALO R *The action of physostigmine, morphine cyclopentolate and homatropine on the secretion and outflow of aqueous humour in the rabbit eye* Acta physiol scand 1972 *86* 239—249

The influence on the intraocular fluid dynamics (intraocular pressure inflow and outflow) of one i v injection or a 3 day topical application of physostigmine morphine cyclopentolate or homatropine was investigated Special attention was paid to the inflow of aqueous humour and ATPase activity in the ciliary body iris after administration of these drugs Systemic injection of physostigmine caused a marked decrease in intraocular pressure which seemed to be due to the decrease in inflow of aqueous humour On the contrary three day topical applications of this drug had no effect on the IOP or intraocular fluid dynamics Intravenous injection of morphine increased both the inflow rate and the outflow facility of the eye Cyclopentolate and homatropine did not decrease the outflow facility However cyclopentolate applied intravenously or topically increased both the inflow rate and the outflow facility slightly Homatropine intravenously injected had no effect on the intraocular fluid dynamics but applied topically slightly increased the IOP possibly due to the increased inflow of aqueous humour None of these drugs caused any significant change in the NaK ATPase activity in the ciliary body iris Physostigmine and morphine markedly reduced Mg ATPase activity Physostigmine did not decrease Mg ATPase activity when the eye had been sympathectomized 7 days before administrations of the drug It was concluded that the effect of physostigmine on Mg ATPase was possibly mediated by way of the sympathetic nervous system

Key words intraocular pressure adenosine triphosphatase ciliary body parasympathomimetics parasympatholytes

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The drugs which affect the autonomic nervous system and which are also taken to include the miotic mydriatic and cycloplegic agents comprise the most commonly used chemicals in ophthalmology

The mechanism by which epinephrine and other sympathomimetic agents exert their hypotensive effect has not been fully explained (Sears 1966) There is general agreement that adrenaline like substances cause a decrease in the secretion rate (Goldmann 1951 Weekers *et al* 1952 Becker and Ley 1958 Garner *et al* 1959) and that these chemicals also increase the outflow facility in the eye (Ballintine and Garner 1961 Becker *et al* 1961)

Sympatholytic drugs, in spite of their many antagonistic effects with sympathomimetics have been shown to be effective in the treatment of glaucoma However, no generally accepted clinical medication has been developed from this group of drugs. Dibenamine a sympatholytic drug, has occasionally been used in the pre-operative management of acute glaucoma

Parasympathomimetic therapy is a favoured control method for treatment of simple open angle glaucoma Topically administered anticholinesterases are in particular widely used to decrease the intraocular pressure (IOP) (Kronfeld 1966) The decrease in IOP under the influence of anticholinesterases is possibly due to a change in the blood aqueous barrier causing a decrease in the inflow of aqueous humour (Barany 1947) On the other hand it has also been claimed that anticholinesterases decrease the IOP by improving the facility of aqueous outflow (Leopold and Krishna 1963)

Parasympatholytic agents are used as mydriatics for the purpose of cycloplegia or examination of the fundus The effect of mydriatics or cycloplegics on IOP has been discussed in numerous papers (Gordon and Ehrenberg 1954 Kronfeld *et al* 1943 Schimek and Lieberman 1961 Christensen and Pearce 1963) Their effect has been explained as causing an increased IOP by decreasing the outflow facility (Barany and Christensen 1967)

In previous works from our laboratory (Uusitalo and Palkama 1971 Harkonen *et al* 1972) Uusitalo 1972 the correlation of ocular fluid dynamics with denervation or stimulation of the two autonomic nerve systems has been studied Special attention has been paid to the marked effect of the parasympathetic nerves on the secretion of aqueous humour Decreased inflow after parasympathetic denervation may be mainly due to an inhibition of NaK ATPase activity in the ciliary body iris (Uusitalo and Palkama 1971)

The purpose of this work was to study whether drugs acting on the parasympathetic nerves have any influence on the secretion of aqueous humour Two parasympathomimetics—physostigmine and morphine—and two parasymphatolytes—cyclopentolate and homatropine—were selected for the study The effect of these drugs on the intraocular pressure rate of aqueous humour formation and outflow facility and on the ATPase content of the ciliary body iris was measured

Material and methods

The material consisted of 42 male albino rabbits weighing 1—3 kg The animals were anesthetized with urethane (1 0—1 75 g kg) The drug was diluted in distilled water and injected into a marginal ear vein

The IOP was recorded manometrically using a pressure transducer (Statham SP 23 D) in conjunction with a recorder (Metrohm E 478) The technique was a modification of the method described by Sears and Barány (1960) It has been presented in more detail earlier (Harkonen *et al* 1971 The facility of aqueous outflow (C) was measured in the animals by noting the pressure change (ΔP) resulting from a constant rate anterior chamber infusion of isotonic saline

In calculating the facilities it was assumed that there was no change in aqueous secretion due to a changed IOP The importance of this matter has been thoroughly discussed by Sears (1960) Facility calculations were made according to the formula $C = \Delta F/\Delta P$ where ΔF equals the particular infusion rate The values for aqueous inflow were derived from the formula $F = C(P - P')$ where P' (episcleral pressure) was assumed to be a constant 9 mmHg (Kornbluth and Linnér 1955)

Administration of the drugs

The act on of physostigmine morphine cyclopentolate and homatropine on the secretion and out flow mechan sm v ere investigated by administering these drugs both as a topical appl cation on the eye and as an intravenous injection One exception tc this v as morphine v hich v as only given intravenou ly

T p cal appl cations The topically applied drugs were given in the following concentrations 1 physost gmine salisylate 1 cyclopentolate hydrochloride (Cyclogyl® Star) and 2 homatropine hydrobromide (Isopto Homatropine® Alcon) The standard amount was 50 μl applied into the lower conjuncti al sac of the right eye and instilled twice a day for three days The last drop was given 1—4 h before the IOP measurements began This group contained 18 animals

Intra enous inj ctions The intravenously injected chemicals were given in the following doses physostigmine salisylate 0 5 mg/kg morphine hydrobromide 7 5 mg/kg cyclopentolate hydrochlo ride 50 mg/kg and homatropine hydrobromide 0 5 mg/kg In the beginning of the experiment the IOP (P) was measured first in the left (control) eye and then in the right eye After this the drug to be studied was injected into the ear vein v hile the manometric measurements v ere going on The IOP was observed in the right (experimental) eye until a new equil brium pressure was reached This took about 30 min The inflow of the aqueous humour and th e outflow facility were then measured in this eye with a constant rate infusion techn que This group contained 17 animals

I t a enous inject ons of dru s after sympathectomy In this experiment 7 animals were subjected to right superior cervical gangl onectomy under th opentane sodium anesthesia 7 days before injection of physostigmine or cyclopentolate intravenously The intraocular fluid dynamics v ere stud ed as above

ATPase measurements

The same animals whose eyes were used for the manometric measurements of IOP inflow and outflow after treatment v ith autonomic drugs were also u ed for b ochemical analyses They were killed by an intravenous air injection The tv o eyes were immediately enucleated and opened anterio ly the lens and the vitreous body were removed and each cil ary body iris preparation was exci ed as a complete ring The preparations were cut in two halves immediately immersed in l quid n trogen and stored at —50 C The biochem cal ATPase analyses were made fluorometri cally as presented earlier (Harkonen *et al* 1972) The routine assay procedure v as the same except that amytal was excluded from the ncubation med a because of its inhibitory effect on NaK ATPase (Jarnef lt 1962 Harkonen *et al* 1972) Homogenates of the whole ciliary body iris block were incubated at 22 C in 1 ml of re gent containing 50 mM Tris HCl pH 8 1 1 9 mM MgCl 0 5 mM PEP 1 6 mM ATP 0 05 mM NADH and 0 0? BSA When the total ATPas was mea sured th med a contained 10 mM of Na^+ and 30 mM of K^+ When measuring Mg ATPase Na and K^+ were om tted B cause NaK ATPase activ ty cannot be determin d directly in a crude preparat on such as that us d in this study net NaK ATP se v a estimated as the difference be tw en total ATPase and Mg ATPase

St tist c l ev luation

In the prel minary experiments t was soon found that nflow rate and outflow facility could b meas-ured r l ably only once in the same eye If more than on measurement wa don n the same eye the variation was greater than between the r ght and left eye of the same animal The results for IOP and inflow rate and outflow facility after ntravenous injection of various drugs were analysed by using the matched pair t test (Richterich 1968) This n ethod tests whether the differ nce betv een the left control eyes (measured before the d ug wa injected) and the r ght experimental eyes (measured 30 min after the drug was injected) of th sam rabb ts a ies s gnificantly from z ro

In the groups in wh ch top cal applicat ons of drugs w e performed both ey were analysed after three days appl cat on Because it v as po s ble that the drug applied al o influenced the untreated contralateral eye via general blood circulation this was not accepted as a rel able control Thus the results obtained on IOP inflow rate and outflow f cility after a 3 d y appl cat on of drugs were analysed using different types of stat st cal calculations First a compar on wa made of wh ther the diff rence between the control (left) eye and the exper mental (right) eye of the same animal differed s g ficantly from zero (matched pair t te t) Aft ord nary alculat ons it as found that in th homat opine group the left (control) eye had al o been contamin t d by the studied d ug Thus the m an of the experimental eyes as compa ed with the mean of the control group (other nd v duals) of 21 left eyes (De Jonge 1964) In th s study a t te t betw en th t o means was used

Intra enous injection of the drugs nfluences both eye s mila ly P cause the d ffe ences in ATPase act vit s between the two ey of the s me animal were equ l to thos b twe n different nd vi luals both right and left eyes were accept d as exper mental eyes They were ar alyzed tat stically by the t test ATPase activitie afte topical applications e an lyz d l k the stat t cal an lyses of IOP in this group us ng both the match d p ir t t st and the t test between two means

TABLE I A Effect of one intravenous injection of physostigmine morphine cyclopentolate or homatropine on intraocular pressure (IOP) outflow facility (C) and inflow (F) about 30 min after the administration of the drug

Drug	Number of Eyes	IOP mmHg	C µl/min/mmHg	F µl/min
Physostigmine (0 5 mg/kg)				
Control (Left) Eyes	5	18 9±0 8	0 28±0 01	2 74±0 24
Experimental (Right) Eyes	5	14 5±0 6	0 29±0 06	1 63±0 38
Mean Difference		4 4±0 9**	−0 02±0 05	1 11±0 16**
Morphine (7 5 mg/kg)				
Control (Left) Eyes	4	17 5±0 9	0 17±0 02	1 42±0 24
Experimental (Right) Eyes	4	16 6±0 6	0 43±0 06	3 34±0 64
Mean Difference		0 9±1 2	−0 27±0 08*	−1 91±0 81
Cyclopentolate (50 mg/kg)				
Control (Left) Eyes	4	15 8±0 6	0 24±0 06	1 59±0 31
Experimental (Right) Eyes	4	18 7±2 1	0 36±0 11	3 17±0 67
Mean Difference		−2 9±1 8	−0 12±0 15	−1 58±0 80
Homatropine (0 5 mg/kg)				
Control (Left) Eyes	4	16 4±0 5	0 24±0 04	1 76±0 26
Experimental (Right) Eyes	4	17 3±0 7	0 21±0 04	1 70±0 26
Mean Difference		−0 9±0 6	0 03±0 06	0 06±0 45

The control eyes were measured before the injection of the drug The statistical significance between the paired eyes (matched pair t test) is marked * if P <0 05 and ** if P<0 01 Standard errors are those of the mean

TABLE I B ATPase activities in the ciliary body iris after one i v injection of physostigmine morphine cyclopentolate or homatropine

Drug	Number of Eyes	Total ATPase	Mg ATPase	NaK ATPase
		10⁻ µmol/mg prot/min		
Physostigmine (0 5 mg/kg)				
Right and Left Eyes	8	5 22±0 74*	3 38±0 26***	1 84±0 53
Morphine (7 5 mg/kg)				
Right and Left Eyes	8	5 53±0 19**	3 77±0 16* *	1 76±0 10
Cyclopentolate (50 mg/kg)				
Right and Left Eyes	7	6 83±0 49	4 46±0 41	2 37±0 38
Homatropine (0 5 mg/kg)				
Right Eyes	4	7 08 ±0 47	6 48±0 93	0 87±0 34
Control Group	21	7 08±0 46	5 23±0 35	1 86±0 22

The statistical significance has been calculated by comparing both right and left eyes with (other individuals) the control eyes (t test between two means) In the Homatropine group only the right (experimental) eyes were (compared) with the control group The statistical significance has been marked * if P<0 05 ** if P<0 01 and * * if P<0 001 Standard errors are those of the mean

Results

Effect of the drugs on intraocular fluid dynamics

The results for IOP and inflow rate and outflow facility obtained after i v injection or a 3 day topical applications of the autonomic drugs are summarized in Table I A and II A The main observations were

TABLE II A Effect of three day topical applications (twice a day into the right eye) of physostigmine cyclopentolate or homatropine on intraocular pressure (IOP) outflow facility (C) and inflow (F)

Drug	Number of Eyes	IOP mmHg	C µl/min/mmHg	F µl/min
Physostigmine 1				
Control (Left) Eyes	10	18 0±0 9	0 21±0 04	1 69±0 25
Experimental (Right) Eyes	10	17 7±0 9	0 19±0 04	1 40±0 27
Mean Difference		0 3±0 6	0 02±0 04	0 29±0 39
Cyclopentolate 1				
Control (Left) Eyes	3	17 8±1 6	0 15±0 00	1 35±0 24
Experimental (Right) Eyes	3	18 6±0 5	0 29±0 03	2 75±0 17
Mean Difference		−0 7±1 2	−0 14±0 03*	−1 41±0 40
Homatropine 2 %				
Control (Left) Eyes	4	19 8±1 8	0 23±0 04	2 71±0 86
Experimental (Right) Eyes	4	21 6±1 12	0 21±0 01	2 68±0 90
Mean Difference		−1 8±2 0	0 02±0 04	0 03±0 83
No Applications				
Control Group	27	18 1±0 4	0 21±0 01	1 89±0 12

The statistical analyses were done by using as control eyes either the paired eyes or the untreated rabbit eyes (control group) When the former eyes were used as controls the significance has been marked * if P<0 05 (matched pair t test) When the mean of the latter eyes was used the significance has been marked if P<0 05 (t test between two means) Standard errors are those of the mean

TABLE II B ATPase activities in the ciliary body iris after 3 day topical applications (twice a day into the right eye) of physostigmine cyclopentolate or homatropine

Drug	Number of Eyes	Total-ATPase	Mg ATPase 10^{-4} µmol/mg prot/min	NaK ATPase
Physostigmine 1				
Control (Left) Eyes	10	5 81±0 83	4 06±0 58	1 75±0 37
Experimental (Right) Eyes	10	5 45±0 88	3 38±0 47 *	2 28±0 60
Mean Difference		0 37±0 78	0 69±0 35	−0 53±0 59
Cyclopentolate 1				
Control (Left) Eyes	4	5 23±0 31	3 87±0 87	1 37±0 65
Experimental (Right) Eyes	4	4 77±1 41	3 73±0 87	1 17±0 61
Mean Difference		0 46±1 43	0 14±1 55	0 20±0 70
Homatropine 2				
Control (Left) Eyes	3	7 13±0 13	4 99±0 19	2 14±0 26
Experimental (Right) Eyes	3	6 38±0 33	4 90±0 25	1 48±0 40
Mean Difference		0 75±0 24	0 03±0 20	0 65±0 21
No Applications				
Control Group	21	7 08±0 46	5 23±0 35	1 86±0 22

The statistical significance has been calculated by comparing the differently treated (experimental) eyes with the control group (t test between two means The significance has been marked * if P<0 01 Standard errors are those of the mean

Physostigmine The data show that after intravenous injection of physostigmine (0 5 mg/kg) there was a marked decrease in IOP (P<0 01) and a decrease in the inflow of the aqueous humour (P<0 01) These changes were noted when the paired

TABLE III A Effect of one intravenous injection of physostigmine or cyclopentolate on intraocular pressure (IOP) outflow facility (C) and inflow (F) of sympathectomized eye

Drug	Number of Eyes	IOP mmHg	C μl/min/mmHg	F μl/min
Physostigmine (0.5 mg/kg)				
Control (Left) Eyes	2	17.7±1.6	0.31±0.02	2.69±0.65
Experimental (Right) Eyes	2	16.9±0.5	0.17±0.03	1.34±0.31
Mean Difference		0.8±1.1	0.14±0.00*	1.34±0.35
Cyclopentolate (50 mg/kg)				
Control (Left) Eyes	4	18.7±1.5	0.20±0.08	1.52±0.28
Experimental (Right) Eyes	4	16.7±0.9	0.37±0.08	2.76±0.46
Mean Difference		2.1±1.5	0.18±0.06	1.24±0.44

Right cervical ganglionectomy performed 7 days before the experiments. The control eyes were measured before the injection of the drug. The statistical significance between the paired eyes (matched pair t test) is marked * if $P<0.05$. Standard errors are those of the mean.

eyes were compared (the control eye was measured before the drug was injected). The outflow facility was unaffected by physostigmine.

Three days of topical application of physostigmine did not cause a noticeable effect on the intraocular fluid dynamics.

Morphine. When morphine was injected i.v. there was no clear change in IOP but both the inflow and outflow seemed to increase. The latter parameter increased so that this change was probably significant ($P<0.05$).

Cyclopentolate. Cyclopentolate caused a slight increase in IOP, in inflow rate and outflow facility, both after i.v. injection (50 mg/kg) and after a 3 day topical application. The change in outflow facility after topical application was probably significant ($P<0.05$).

Homatropine. Intravenous injection of homatropine (0.5 mg/kg) did not cause any clear change in the intraocular fluid dynamics. A three day topical application resulted in an increased IOP, which seemed to be due to the increased inflow. Increased values (IOP and inflow) were also seen in the control eye, probably resulting from absorption of the drug into the general circulation. When the experimental eyes in this group were compared with the mean of the control group (untreated animals), the increase in IOP and inflow were probably significant ($P<0.05$). The outflow facility was unaffected by topical homatropine.

Effect of the drugs on the ATPase activity of the ciliary body iris block

None of the drugs studied had any effect on the NaK ATPase activity of ciliary body iris, as presented in Table I B and II B. On the other hand, two of the drugs caused a decrease in Mg ATPase activity.

After i.v. injection of physostigmine or morphine, Mg ATPase activity decreased highly significantly ($P<0.001$) as compared with the mean of the control group (untreated animals).

TABLE III B ATPase activities in the ciliary body iris of sympathectomized eye after one i v injection of physostigmine morphine or homatropine

Drug	Number of Eyes	Total ATPase	Mg ATPase	NaK ATPase
		10^{-} / mol/mg prot/min		
Physostigmine (0 5 mg/kg)				
Experimental (Right) Eyes	3	6 93±1 73	5 69±0 88	1 64±0 59
Cyclopentolate (50 mg/kg)				
Experimental (Right) Eyes	4	7 08±0 48	6 30±0 27	0 80±0 38
Control Group	21	7 08±0 46	5 23±0 35	1 86±0 22

The statistical analyses revealed no significant differences between the experimental (sympathectomized) eyes and the control group (other individuals t test between two means) Standard errors are those of the mean

After topical application of physostigmine Mg ATPase activity in the ciliary body iris decreased significantly (P<0 01) as compared with the mean of the control group Topical application of the drugs evidently also influenced the enzyme in the control (contralateral) eye

Effect of the drugs on intraocular fluid dynamics and ATPase activity after sympathectomy

The effect of physostigmine and cyclopentolate on the denervated eye was investigated by intravenous injection of these drugs It was seen that the effect on IOP and intraocular fluid dynamics was about the same as on a normal intact innervated eye Physostigmine caused a decrease in IOP and inflow rate Cyclopentolate increased both inflow rate and outflow facility as noted above (Table III A)

I v injected physostigmine did not change the Mg ATPase activity in the ciliary body iris of the denervated eye (Table III B) It ought to be remembered that in un-denervated animals this drug decreased Mg ATPase activity more than 40 % Cyclopentolate also had no effect on Mg ATPase activity in the denervated eye (Table III B)

Discussion

The decrease in IOP after systemic physostigmine seems to be due to the decreased inflow of aqueous humours On the other hand a three day topical application of this drug did not alter the intraocular fluid dynamics The temporary hypotonia characterizing systemic physostigmine action in the present study is in good agreement with the findings of Larsson (1930) and can be explained by the fall in osmotic pressure in the eye (Barány 1947) The decrease in aqueous inflow after i v physostigmine may also be partly due to the increased blood aqueous barrier system and is perhaps also a result of the decreased blood supply to the anterior uvea The decrease in blood flow might well result from a direct compression of the blood vessels in the uveal region secondary to physostigmine induced ciliary muscle contraction

As physostigmine only had a temporary effect on the intraocular fluid dynamics and

the NaK ATPase activity in the ciliary body iris did not change it is likely that the drug had no effect on the active secretory mechanism This is in disagreement with the works of Berggren (1965) whose *in vitro* studies have shown that physostigmine is an effective inhibitor of the aqueous secretion mechanism The discrepancy between the present results and those of Berggren may be explained partly by the different methods and partly because the NaK ATPase activity is not the only indication of the secretory activity of the ciliary body

The significant decrease in Mg ATPase activity in the ciliary body iris after both systemic and local application of physostigmine is not likely to have had any effect on the intraocular fluid dynamics Because the changes were so significant the possible mechanism by which physostigmine decreases the Mg ATPase activity is discussed

The effect of physostigmine is commonly described as potentiating the action of acetylcholine by inhibiting acetylcholinesterase The dependence of the anticholinesterase action upon intact nerves has been demonstrated by Anderson (1955) and Wescoe and Riker (1951) It has thus been concluded that physostigmine exerts its pharmacologic effect solely as a result of its anticholinesterase activity However many investigators have provided evidence for the existence of a non anticholinesterase action of physostigmine (Varagic 1955 Leslić and Varagic 1961 Varagić *et al* 1967 Stamenovic and Varagic 1970) They have shown that physostigmine treatment leads to an increased efferent activity in the cervical sympathetic system and also to general sympathetic activation

The adrenergic nervous system has a unique role in controlling rapid changes in the mobilization of energy fuel by glycogenolysis and lipolysis (Brodie *et al* 1966) Catecholamines trigger the activation of liver phosphorylase which catalyses the breakdown of glycogen to glucose 1 phosphate or glucose (Sutherland and Rall 1960) The marked decrease in Mg ATPase activity in ciliary body iris after physostigmine treatment may well be the corresponding metabolic effects following adrenergic activation This would explain why Mg ATPase activity in ciliary body iris did not decrease when the eye was sympathectomized before physostigmine treatment

In order to confirm that the effect of physostigmine on Mg ATPase in the ciliary body iris was indirect some *in vitro* studies were performed Homogenates of normal ciliary body iris were incubated at 22 C in the presence of physostigmine for half an hour Decrease in ATPase activity was not seen (Uusitalo unpublished observation) This has also been shown by Jarnefelt (1961)

One i v injection of morphine caused an increase in both inflow rate and outflow facility The IOP did not change reflecting the fact that the effects of morphine on the inflow and outflow mechanisms were compensatory This is in disagreement with the finding of Leopold and Comroe (1948) that morphine decreased IOP They have, however measured IOP only using a Schiotz tonometer and did not record inflow rate or outflow facility

The increase in inflow rate and outflow facility due to morphine may be explained by the reduced control of these two mechanisms by both the sympathetic and the parasympathetic nerves This can be explained on the basis of the findings indicating

that morphine not only inhibits the release of acetylcholine (Paton 1957, Schaumann 1957 Beleslin and Polak 1965 Hano *et al* 1964) but also the release of a sympathetic transmitter from the postganglionic nerve endings (Trendelenburg 1957 Cairnie *et al* 1961)

The NaK ATPase activity was unaffected after morphine injection This favours the opinion that the morphine effect only influences a passive mechanism in the aqueous inflow

The effect of morphine in decreasing Mg ATPase activity, seen in the present study seems to be a direct effect In vitro incubation of 10^{-6} M morphine with normal ciliary body iris homogenate for 1/2 hr clearly decreased Mg ATPase activity (Uusitalo unpublished observation)

Cyclopentolate and homatropine did not decrease the outflow facility This is contradictory to many other reports claiming that these drugs decrease the outflow facility (Christensen and Pearce 1963 Bárány and Christensen 1967) The IOP stayed unchanged or increased slightly Similar findings have been reported by Kollner (1921) Kronfeld *et al* (1943) Barbee and Smith (1957)

The effect of cyclopentolate increasing both the inflow rate and the outflow facility may be explained by the reduced parasympathetic control of both mechanisms Topical application of homatropine also increased the inflow which can be explained by the reduced nervous control of the inflow mechanism It has been shown earlier that stimulation of the parasympathetic nerve causes a clear increase in the inflow of aqueous humour, probably by increasing the permeability of the blood aqueous barrier (Uusitalo 1972) Ciliarectomy or coagulation of the parasympathetic nerves resulted in a decreased inflow (Harkonen *et al* 1972) In the light of these experiments it seems difficult to explain the effects of cyclopentolate and homatropine which are parasympatholytic blocking agents It is probable that the barrier mechanism in the eye is regulated in different ways The same agents which affect the barrier through the nervous system may have a different effect when acting directly on the barrier

Atropine has an anomalous vascular effect, producing dilatation of the cutaneous vessels (Goth 1966) On the other hand there is no known action of atropine on neural transmission which could explain the dilatation of the blood vessels Homatropine and cyclopentolate may have the same kind of vasodilating effect as atropine on the uveal blood vessels resulting in increased permeability of the blood aqueous barrier and an increase in the inflow of aqueous humour

Whether the drugs caused an increase or decrease in the episcleral pressure (P_v) remained unsolved When the present results concerning the inflow are inspected one must remember that the calculations are based on the assumption of a constant episcleral pressure (9 mmHg)

Intravenously injected physostigmine decreased the inflow On the contrary morphine cyclopentolate and homatropine tended to increase the inflow of the eye Thus the drugs studied clearly have an effect on the inflow mechanism of the aqueous humour, but not on the outflow NaK ATPase activity in the ciliary body iris was unchanged by these drugs On the basis of the present findings it can be concluded

that the effect on the intraocular fluid mechanism of the drugs studied cannot be explained purely as stimulatory or inhibitory effects on the parasympathetic nerves The present results differed markedly from those obtained when parasympathetic nerves were either electrically stimulated or denervated by coagulation (Uusitalo and Palkama 1971 Uusitalo 1972) Although the mechanism by which the parasympatholytic and mimetic drugs influence intraocular fluid dynamics remains open it can be said that inflow seems to play a more important role than outflow

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Lipid Biosynthesis from DL (2-^{14}C) Mevalonic Acid in Intact Mice and Rabbits

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Abstract

Ldgren B and K Hellstrom *Lipid biosynthesis from DL (2-^{14}C) mevalonic acid in intact mice and rabbits* Acta physiol scand 1972 86 250—256

DL-mevalonate 2 ^{14}C was administered parenterally to 2 rabbits and 5 groups of mice In the rabbit the amount of labelled material recovered in the non saponifiable lipids of the kidneys exceeded that of the liver Most of the renal radioactivity was found in the squalene lanosterol and methostenol fractions whereas the major part of the labelled material in the liver was present as radioactive cholesterol The distribution of radioactivity within the kidney and the liver in the mice varied with the size of the administered dose The smaller the dose the larger the proportion of label recovered in the kidneys In all experiments most of the radioactivity of the liver was transformed to cholesterol The conversion of squalene to cholesterol proceeded more slowly in the kidneys and 30 min after the administration of the mevalonate substantial amounts of radioactivity was recovered as labelled squalene and lanosterol The importance of circulating mevalonate as substrate in the cholesterol synthesis of the kidney will be discussed

The metabolic sequence that results in the formation of cholesterol includes a number of intermediates such as acetoacetate 3 hydroxy 3 methylglutaric acid mevalonic acid and squalene a hydrocarbon with 30 carbon atoms Squalene is transformed to lanosterol which is converted to cholesterol via several intermediates Mevalonic acid appears to have a key position in the regulation of these processes Although cholesterol feeding influences several steps in this synthetic pathway (Gould and Swyryd 1966) the inhibition of the transformation of 3 hydroxy 3 methylglutaric acid to mevalonic acid appears to be the primary site of the negative feedback inhibition of the cholesterol synthesis in the liver (Siperstein and Fagan 1966)

A recent study in normal rats (Hagenfeldt and Hellstrom 1972) demonstrated the presence of a circulating pool of mevalonate A major part of this mevalonate is taken up by the kidneys and transformed into non saponifiable lipids (Hellstrom *et al* 1972) Since the circulating mevalonate may be an important substrate in the renal sterol synthesis it was considered of interest to study whether similar observa

tions could be made in other species The aim of the present investigation was to study the metabolic fate of labelled mevalonate administered parenterally to intact mice and rabbits

Material and Methods

Animals Female white mice (average weight 20 g) and 2 female rabbits (average weight 3 1 kg) were fed ground pellet food *ad libitum* before the experiments

Experimental procedure Radioactive mevalonate was administered i p and i v in doses of 0 015—0 3 and 0 022—0 90 µmol respectively to unanesthetized mice The animals were sacrificed after 0 5 1 2 4 or 24 h The liver kidney and intestines were taken out immediately and placed in chloroform methanol (1 1) The rabbits were studied under urethane anesthesia They received 5 2 µmol of labelled mevalonate by an i v injection The rabbits were killed by exsanguination via an aortic catheter after 110 and 155 min respectively

Preparation of labelled mevalonate DL-mevalonic acid 2 ¹⁴C lactone (4 82 mCi/mmol) was obtained from the Radiochemical Centre Amersham England The labelled material was dissolved in saline and converted into the potassium salt as described by Goodman and Popjak (1960) Various doses of mevalonate were prepared by addition of unlabelled mevalonate The radiopurity of the mevalonic acid lactone was checked by thin layer chromatography (TCL) on silica gel G using chloroform methanol (7 3) as solvent system No impurities were detected by autoradiography of the chromatograms

Analytical methods The tissues were cut into small pieces and extracted with chloroform methanol (1 1 v/v) at boiling temperature for two hours Aliquotes of the extracts were saponified in alkaline dioxane (Eneroth and Nystrom 1967) The unsaponifiable lipids were extracted three times with light petroleum ether each portion being washed twice with a small volume of 50 % ethanol The combined extracts weer taken to dryness *in vacuo* with a rotary evaporator and transferred to a small volume of chloroform

In some experiments the tissues were analyzed for allyl pyrophosphates and prenoic acids as described by Goodman and Popjak (1960) The saponification was then performed in M KOH in 50 % ethanol at 60 C for 1 h The non saponifiable lipids were extracted with light petroleum ether as described above The water phase was then acidified On the next day the aquous phase was again made alkaline and the hydrolyzed prenoic alcohols were extracted with light petroleum ether The prenoic acids were obtained after final acidification of the aquous phase and repeated extractions with light petroleum ether

The non saponifiable lipids were fractionated by TLC on silica gel coated plates With ethyl acetate benzene 1 5 as solvent system (Avigan *et al* 1963) the material is separated into the following bands Squalene (Rf 0 94) nerolidol (Rf 0 72) C 30 sterols *i e* lanosterol and dihydrolanosterol (Rf 0 63) farnesol and C 28 sterol *i e* methostenol (Rf 0 54—0 48) geraniol and C 27 sterols presumely cholesterol (Rf 0 40) Squalene lanosterol and cholesterol (about 20 µg of each) were spotted together with the extract to be chromatographed in 2 5 cm bands The unsaponifiable lipids were checked for the presence of sterol esters by TLC using heptane benzene 4 1 as solvent system The spots were detected under UV light on the plates that had been sprayed with 0 003 % Rhodamine G solution in 50 % ethanol The gel with the visible bands as well as those from the interposed areas were scraped off separately Each fraction was transferred to a counting vial containing liquid scintillation fluid (0 3 % diphenyl oxazole and 0 015 per cent p bis phenyl oxazolyl benzene in toluene) The radioactivity was counted in a Packard liquid scintillation counter An internal standard was used to correct for quenching

Results

Mice experiments 30 min after an i v injection of 0 022 µmol of labelled mevalonate the amount of radioactivity recovered in the kidney (30 per cent) was about 3 times higher then that of the liver This difference became more pronounced during the next hours since the radioactivity in the liver decreased more rapidly than that of the kidney (Fig 1) With a considerably larger dose of mevalonate (0 9 µmol) the 30 min recovery was 8 per cent in the kidneys and approximately the same in the liver At 1—4 h the kidneys contained more radioactivity than the liver (Fig 2)

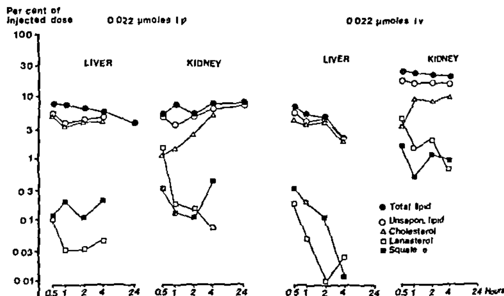


Fig 1 Recovery (per cent of given dose) after i v and i p injection of a small tracer dose of DL-mevalonate 2 [14]C to mice

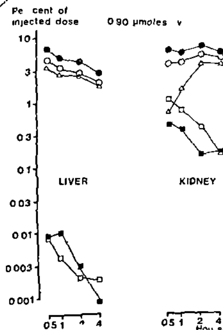


Fig 2 Recovery (per cent of given dose) after injection of a large tracer dose of DL-mevalonate 2 [14]C to mice

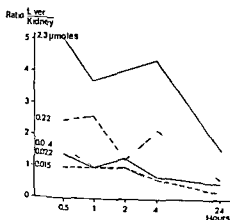


Fig 3

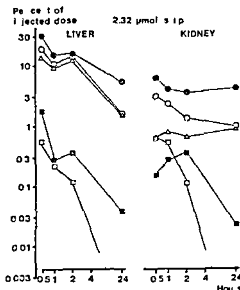


Fig 4

Fig 3 Ratio between the total amounts of radioactivity recovered in the liver and kidneys after i p injections of different doses of DL mevalonate 2 ^{14}C to mice

Fig 4 Recovery (per cent of given dose) after i p injection of a large tracer dose of DL mevalonate 2 ^{14}C to mice

I p administration of labelled mevalonate was employed in 5 groups of mice With increasing doses of mevalonate proportionally more radioactivity was recovered in the liver than in the kidneys (Fig 3) The dose dependence of the distribution of the labelled substance was most pronounced at 30 min but it was evident even after 24 h At 30 min the uptake in the kidney equalled that of the liver only in the mouse that received the smallest amount of mevalonate (0.015 μmol)

In both liver and kidneys the major part of the radioactivity was present as non saponifiable material This finding was independent of the size of the dose of mevalonate and mode of administration In both organs most of the non saponifiable radioactivity accumulated in the squalene the C 30 sterol (lanosterol) and the C 27

TABLE I Recovery of total and non saponifiable ^{14}C metabolites following i administration of DL mevalonate 2 ^{14}C to rabbits (per cent)

^{14}C-metabolites	Liver		Kidney		Blood	
	Total	Non sap	Total	Non sap	Total	Non sap
Rabbit 1	6.1	4.2	9.3	8.6	2.8	1.0
Rabbit 2	5.5	3.1	9.6	6.8	2.4	1.0

Total blood volume calculated as 1/17 of the body weight

TABLE II Distribution of the nonsaponifiable lipids of the rabbits in squalene (Sq) C 30 C 28 and C 27 sterol fractions Per cent

Rabbit	Liver				Kidney				Blood			
	Sq	C 30	C 28	C 27	Sq	C 30	C 28	C 27	Sq	C 30	C 28	C 27
1	3	2	2	82	13	34	10	9	2	2	3	68
2	3	2	1	79	35	37	9	9	1	2	3	82

sterol (cholesterol) bands of the thin layer chromatogram However the two organs differed markedly with regard to the pattern of distribution of labelled material within these bands (Fig 1 2 and 4) In the liver 70—80 per cent of the non saponifiable label was present as C 27 sterol presumably as cholesterol already at 30 min The amounts of radioactive squalene and lanosterol decreased rapidly and only trace amounts were found after 4 and 24 h The transformation of mevalonate into cholesterol proceeds more slowly in the kidney In several experiments there was more label in the lanosterol than in the cholesterol band at 30 min As a rule the kidneys contained more radioactive squalene than the liver Both the liver and the kidneys contained only minor amounts of labelled allyl pyrophosphates and prenoic acids These fractions were not further analyzed

The gastrointestinal tract contained 9—16 per cent of administered radioactivity Only 1/4—1/3 of that label was extractable with light petroleum ether The pattern of distribution of label among the squalene C 30 and C 27 sterol bands was essentially the same as in the liver

Rabbit experiments In the rabbit that was killed after 110 min 4 2 8 6 and 1 0 per cent of the administered radioactivity was recovered as non saponifiable lipids in the liver kidneys and total blood volume (calculated as 1/13 of the body weight) respectively The corresponding figures for the animal that was killed after 155 min was 3 1 6 8 and 1 0 per cent (Table I) Cholesterol was the major labelled non saponifiable lipid of the liver which also contained minor amounts of labelled squalene C 30 and C 28 sterols (Table II) Most of the radioactive non saponifiable lipids in the kidneys was recovered as squalene and lanosterol whereas in both animals only 9 per cent of this radioactivity was localised to the cholesterol band The label in the non saponifiable lipids of the blood was distributed in a similar way as that in the liver (Table II)

Discussion

In vitro studies in the rat demonstrated that the kidney in comparison with the liver, had a low capacity to synthesize mevalonate from acetate (Hellstrom *et al* 1972) The efficacy by which mevalonate was transformed into non saponifiable lipids was about the same in both tissues Squalene and lanosterol were the major

end products in the kidney whereas most of the non saponifiable radioactivity in the liver was recovered as labelled cholesterol Upon parenteral injection of approximately 75 μg of labelled mevalonate the uptake of radioactivity by the kidneys was far higher than the combined amounts recovered in all other tissues studied When the amount of administered mevalonate was increased to 1 mg almost equal amounts of radioactivity was recovered in the kidneys and the liver Most of the label was transformed to non saponifiable lipids but in similarity with the findings *in vitro* the rate of the conversion of squalene and lanosterol to cholesterol appeared to be slow in the kidney Up to 4 h after parenteral administration of DL mevalonate 2 ^{14}C most of the renal radioactivity was distributed to the squalene and lanosterol fractions

The present study shows that the metabolic fate of i v administered mevalonate in the rabbit is similar to that previously observed in the rat The uptake in the kidneys exceeded that of the liver The major part of the renal radioactivity was incorporated into the cholesterol precursors squalene lanosterol and probably methostenol and only small amounts of label was recovered in the cholesterol fraction

The distribution of label among the kidney and the liver of the mice was very much dependent upon the amount of the mevalonate administered With the small intravenous dose (0 022 μmol) the ratio between the kidney and the liver radioactivity was approximately 3 After an i v injection of 0 9 μmol the liver contained about the same amount of label as the kidney Five doses of mevalonate were employed for i p administration to the mice Each increase of the dose was followed by a proportional elevation of the label recovered from the liver The rate of the transformation of squalene and lanosterol to cholesterol in the kidney was more rapid in the mice than in the rabbits and the rats However when compared to the liver the rate of these processes was slow in the kidney

A recent report (Hagenfeldt and Hellstrom 1972) demonstrated that the concentration of mevalonate in rat blood was 0 02—0 04 μg per ml On the basis of these figures it appears that the fate of an injected dose of 75 μg rather than 1000 μg of labelled mevalonate to a rat was reflecting the metabolism of the circulating acid of endogenous origin DL mevalonate was used in the studies referred to above Since the inert D isomer is excreted nonmetabolized in the urine (Gould and Popjak 1957) a substantial part (up to 60 per cent) of the L form was used in the renal synthesis of non saponifiable lipids

The results from the present study indicate that the situation is similar in mice and rabbits Thus kidneys of the mice that received the smallest i v dose of mevalonate showed the highest recovery of radioactivity In the rabbits the amount of radioactive renal lipids was relatively small but the injected dose was high and may have expanded the circulating mevalonic acid pool considerably

In vitro studies have shown that most tissues are able to synthesize cholesterol from acetate (Dietchy and Siperstein 1967) but the relative contribution of circulating cholesterol precursors to the cholesterol synthesis in the various tissues of

the intact animal is not established Small amounts of squalene and lanosterol (Goodman 1963) have been detected in the blood of the rat The previous studies in rats (Hellstrom *et al* 1972, Hagenfeldt and Hellstrom 1972) showed that circulating mevalonate may be an important substrate in the renal synthesis Whether the blood mevalonate could be of the same importance in mice and rabbits can not be predicted until the plasma concentration and the turnover rate of the circulating mevalonate is known

The mechanisms that regulate the formation of cholesterol in extrahepatic tissues is poorly understood The hepatic synthesis that contributes a considerable part of the serum cholesterol of endogenous origin is markedly reduced after cholesterol feeding (Siperstein and Fagan 1966) An inhibition of the cholesterol synthesis was not observed in the 16 extrahepatic tissues of the rat studied *in vitro* with labelled acetate (Dietschy and Siperstein 1967) Most of the circulating mevalonate probably originates from the liver and the secretion of newly synthesized mevalonate into the blood is markedly reduced upon cholesterol feeding (Hagenfeldt and Hellstrom 1972) It appears then that the negative feedback system in the liver may influence the synthesis of sterols in the kidney by inhibiting the formation of the circulating mevalonate

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In Vitro Work Load and Rat Heart Metabolism

II Effect on amino acid transport

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Abstract

Ahren K Å Hjalmarson and O Isaksson *In vitro work load and rat heart metabolism II Effect on amino acid transport* Acta physiol scand 1972 86 257—270

In vitro pressure overload was found to accelerate protein synthesis in the isolated working rat heart (Hjalmarson and Isaksson 1972 a) Since membrane transport of amino acids is considered to be one rate limiting step in protein synthesis the amino acid transport in the isolated rat heart was investigated using the non utilizable amino acids α aminoisobutyric acid (AIB) and 1 aminocyclopentane carboxylic acid (cycloleucine) Increased pressure load (afterload) accelerated amino acid uptake after a perfusion period of 15 min and a 50—100 % increase in the intracellular to extracellular distribution ratio of the amino acids was seen after 60 min of perfusion Thus accelerated uptake was not inhibited by cycloheximide suggesting that the work load effect was not dependent upon a continuous synthesis of proteins The accumulation rate continued to be stimulated for some time after normalization of the work load and coronary flow indicating that the work load effect was not directly linked to coronary flow Increased preload did not stimulate amino acid uptake Hearts from hypophysectomized rats showed a decreased concentrative uptake but the amino acid uptake was still accelerated by pressure overload It is suggested that an increased uptake of amino acids could be of physiological significance in relation to the increased protein synthesis under these conditions

In a preceding paper it was reported that increasing the afterload of the isolated rat heart could accelerate the incorporation of phenylalanine into the whole heart protein (Hjalmarson and Isaksson 1972 a) This effect was seen after a perfusion period of only 30 min An increase in the preload did *not* give such an effect although the external cardiac work was increased three times It was concluded from this study that an increase in contractile tension during systole and/or an increase in the end systolic volume was a necessary requirement for the acceleration of protein synthesis Mechanisms through which the increased afterload of the heart accelerates the protein synthesis were not studied in the preceding paper (Hjalmarson and Isaksson 1972 a)

Changes in the work load can theoretically affect several rate limiting steps in protein synthesis 1 membrane transport of amino acids 2 intracellular amino acid activation 3 transcription processes and 4 translation processes at the ribosomes

Correlation between the rate of amino acid transport into tissues and the rate of tissue growth or amino acid utilization is generally considered to be good (Christensen 1962 Riggs 1964) It has been reported that the uptake of amino acids is increased in hypertrophying skeletal and cardiac muscles (Goldberg and Goodman 1969 Wannemacher and McCoy 1969) Moreover, the uptake of the non utilizable amino acid *α* aminoisobutyric acid (AIB) is increased in skeletal and cardiac papillar muscles by increased mechanical activity *in vitro* (Arvill 1967, Lesch *et al* 1970 Goldberg 1971)

It has been shown that the intracellular concentrations of several amino acids are very constant when the rate of protein synthesis in the isolated heart is markedly changed (Hjalmarson *et al* 1970 Morgan *et al* 1971 a b Jefferson *et al* 1971) This observation could be an argument against the significance of increased amino acid transport in regulation of heart protein synthesis However measurements of the intracellular concentrations of natural amino acids do not mirror the rate of membrane transport of amino acids since the intracellular free amino acids are readily build up into tissue proteins and utilized The role of the intracellular pool of free amino acids as an intermediate in protein synthesis has also been questioned (Waterlow and Stephen 1968 Hider *et al* 1969) On the other hand such a role by the amino acid pool could be supported by the observation that lysine glycine and phenylalanine equilibrated with the intracellular pool of amino acids before the incorporation into tissue proteins of the perfused rat heart (Morgan *et al* 1971 a)

The present experiments were undertaken to investigate the effects of well defined work loads on the amino acid transport in the isolated working rat heart using the non utilizable amino acids AIB and 1 aminocyclopentane carboxylic acid (cycloleucine) Some preliminary results of these experiments have been published in abstract form (Hjalmarson and Isaksson 1969)

Methods

Experimental procedure

Hearts were removed from normal and hypophysectomized fed female rats of the Sprague-Dawley strain (170—230 g) Hypophysectomy was performed on rats weighing about 200 g by the standard parapharyngeal approach The body weights of the hypophysectomized rats decreased 10—20 g during the 2 weeks from the operation to the time of the experiment The rats were anesthetized with Nembutal (60 mg/kg b w) and the hearts were rapidly excised and transferred to ice chilled saline The aorta and the left atrium were cannulated and after a retrograde washout period of 5 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) the hearts were perfused in the forward direction as described previously (Neely *et al* 1967 a Hjalmarson and Isaksson 1972 a) The perfusion medium was Krebs Henseleit bicarbonate buffer containing the disodium salt of EDTA (0 5 mM) with glucose (14 mM) or palmitate (1 5 mM) as substrate When incorporation of phenylalanine ^{3}H into tissue protein was studied the buffer contained all amino acids at normal rat plasma levels (Morgan *et al* 1971 a) The recirculating perfusion volume was 40 ml during

the subsequent periods The temperature of the perfusate was maintained at 37 C and the buffer was continuously gassed with 95 % O_2 and 5 % CO_2 equilibrated with water at 37 C Th control hearts were perfused at a left atrial filling pressure of 10 cm H_2O and the hearts were forced to pump the perfusate against a hydrostatic pressure of 70 cm H_2O The pressure overloaded hearts were perfused under the same conditions with the difference that the aortic tube was totally constricted The peak systolic pressure in the aortic tube was increased from about 95 mm Hg to about 160 mm Hg by this constriction There was a concomitant increase in the aortic diastolic pressure from approximately 45 to 120 mm Hg and an increase in the coronary flow from 75 to 200 ml/g dry weight×min The heart rate was about 240 beats/min with no difference between the control and the overloaded hearts Aortic pressures and heart rate were recorded by a transducer attached to a manifold of the aortic tube and the pressure tracings registered by a Sanborn recorder model 16—1300 S Coronary flow was measured b collecting the fluid dropping from the heart

Chemicals

The radioactive substances were obtained from New England Nuclear Co Boston USA The substances were used with the following specific activities and molarities α aminoisobutyric acid ^{3}H(G) (AIB ^{3}H) 0 4 μCi/μmol 0 05 mM 1 aminocyclopentane 1 carboxylic acidcarboxyl ^{14}C (cycloleucine ^{14}C) 0 04 μCi/μmole 0 1 mM L-phenylalanine ^{3}H (G) 1 25 μCi/μmole 0 08 mM sucrose ^{14}C (U) 3 26 μCi/μmole 0 01 mM Cycloheximide was purchased from Nutritional Biochemicals Co Cleveland USA

Determination of the intracellular accumulaton of AIB ^{3}H and cycloleucine ^{14}C

At the end of the perfusion period the hearts were cut in a beaker containing ice chilled saline About 250 mg of the lateral wall of the left ventricle was excised rinsed rapidly in buffer weighed and homogenized in 5 % perchloric acid (PCA) The intracellular accumulation of the substances is expressed as the distribution ratio of radioactivity between the intra and extracellular compartments (cpm/ml intracellular water cpm/ml medium) as described previously (Hjalmarson and Isaksson 1972 a) Double labelled samples were counted in a Tri Carb Packard Model 3375 spectrometer with settings which gave a 10—15 % overlap of ^{14}C radioactivity in the ^{3}H channel and a 1 % overlap of ^{3}H activity in the ^{14}C channel The two isotopes (cycloleucine-^{14}C and AIB-^{3}H) were counted with an efficienc of about 4 % for ^{3}H and 60 % for ^{14}C The standard error was less than 2 % for the determination of sucrose ^{14}C AIB-^{3}H and cycloleucine ^{14}C The degree of quenching was tested b automatic external standardization in each sample and it as found to be the same for the tissue extracts and their respective perfusion media It was therefore not necessary to correct the counting data for quenching prior to the calculations of the extracellular paces and distribution ratios The extra and intracellular spaces of hearts from normal rats perfused at different experimental conditions have been presented in a previous paper (Hjalmarson and Isaksson 1972 a) Perfusion of the hearts with cycloheximide did not change the spaces significantl In hearts from hypophysectomized rats the total tissue water was 83 8±0 2 ml/100 g et heart and the extracellular space determined with sucrose ^{14}C 36 5±1 1 in hearts perfused under control conditions The corresponding values for hearts from hypophysectomized rats perfused with pressure overload was 85 1±0 9 and 44 2±1 3 ml/100 g wet heart respectively There were 4 hearts in each group and the hearts were perfused for 60 min

Statistics

Analysis of variance (with one criterion of classification) followed by the Student Newman Keuls multiple range test was used (Woolf 1968) A simple t test was used to compare groups IX and X in Fig 2 which had variances significantly lower than for all other groups A p-value of 0 05 or less was considered significant in this study

Results

Effects of increased pressure load (increased afterload) on the accumulation of AIB ^{3}H and cycloleucine ^{14}C

A time course study of the effect of increased afterload on the accumulation rate of AIB and cycloleucine in the perfused working rat heart was performed as shown by Fig 1 It can be seen from the figure that increased pressure load stimulated

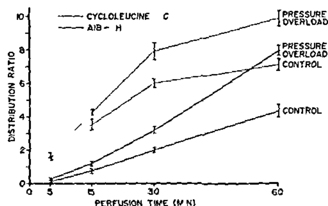


Fig 1 Effects of increased pressure work load on the accumulation of AIB ^3H and cycloleucine-^{14}C by perfused working rat heart Hearts were perfused for various periods with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) with and without pressure overload Each point represents a mean of 4–7 observations and standard errors are indicated by vertical lines. Effects of overload are significant after 15 and 30 min of perfusion for AIB ^3H and cycloleucine-^{14}C respectively ($p < 0.05$)

the accumulation of both AIB and cycloleucine For AIB a significant effect of increased afterload was seen after only 15 min of perfusion, while a significant effect on cycloleucine distribution was seen after 30 min The kinetics for the concentrative uptake of AIB and cycloleucine were quite different The intracellular concentration of AIB continued to increase at a nearly linear rate for the whole perfusion period in both control and pressure overloaded hearts and there was no sign of levelling off to a steady state plateau For cycloleucine there was an initial rapid uptake during the first 30 min of perfusion followed by a slower phase during the subsequent 30 min in the two groups of hearts

Since it has been shown that the rate of protein synthesis in the heart is dependent upon the substrate supply and the availability of amino acids (Morgan *et al* 1971 a Hjalmarson and Isaksson 1972 a) the effect of pressure overload on the uptake of AIB was studied in presence of various substrates and with and without all amino acids at normal plasma levels as shown in Fig 2 It can be seen from the figure that increased afterload stimulated the uptake of AIB in hearts perfused for 30

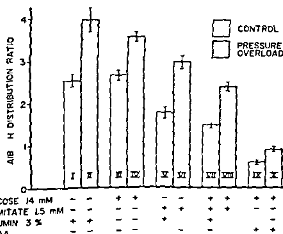


Fig 2 Effects of increased pressure work load on the intracellular accumulation rate of AIB ^3H in presence of various *substrates Hearts* were perfused for 30 min with and without pressure overload with Krebs-Henseleit bicarbonate buffer containing substrates as indicated in the fig There are 4–6 hearts in each group and the standard error is indicated at the top of each bar 1×AA = all amino acids at normal plasma levels

I vs II III vs IV V vs VI I
VII vs VIII IX vs X I
III vs V VII II IV vs VI
VIII VI vs VIII $p < 0.05$
V vs VII N S

TABLE I Effects of increased pressure work load on accumulation of AIB-³H and cycloleucine ¹⁴C after normalization of pressure load on heart

Perfusion period							
0 to 60 min		60 to 90 min					
Experimental condition	Coronary flow ml g d w × min	Experimental condition	Coronary flow ml g d w × min	AIB-³H distribution ratio		Cycloleucine ¹⁴C distribution ratio	
Control (6)	81 ± 4	Control	83±5	2 30±0 14	p<0 05	5 37±0 16	p<0 05
Pressure overload (6)	231±11	Control	79±6	3 86±0 24[b]		6 0±0 37[f]	
Control + cycloheximide 10⁻⁴ M (6)	85 ± 6	Control + cycloheximide 10⁻⁴ M	83±6	1 19±0 10[c]	p<0 05	3 95±0 39[g]	p<0 05
Pressure overload + cycloheximide 10⁻⁴ M (6)	214±18	Control + cycloheximide 10⁻⁴ M	84±3	2 36±0 18[d]		5.23±0.24[h]	

Hearts were perfused for 90 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) The labelled amino acids were added to the perfusion medium after an initial perfusion period of 60 min with (pressure overload) and without (control) aortic constriction to make a final molarity of 0 05 mM for AIB and 0 1 mM for cycloleucine Values are means±S E Number of hearts in each group is indicated in parentheses a vs c b vs d e vs g f vs h $p<0.05$

min both when glucose palmitate or palmitate and glucose in combination were used as substrates Increased afterload also accelerated the uptake of AIB in absence of any exogenous substrate or when all amino acids at normal plasma levels were added to the perfusion medium It can further be seen from the figure that palmitate significantly reduced the rate of uptake of AIB both in the control and overloaded hearts compared to the situation when glucose was present in the perfusion medium When glucose and palmitate in combination were added to the perfusion medium the distribution ratio for AIB was further decreased in the pressure overloaded hearts The inhibition of the membrane transport of AIB by palmitate was independent of the presence of albumin in the perfusion medium since albumin *per se* did not depress the uptake of AIB (Fig 2) The accelerated AIB uptake in the pressure overloaded heart in absence of any exogenous substrate demonstrates that an increase in the uptake of glucose or palmitate associated with increased pressure development (Neely *et al* 1969) is not an obligatory demand for the increased AIB-uptake The addition of all normal amino acids at normal plasma levels to the perfusion medium markedly decreased the distribution ratio of AIB (Fig 2) This effect is probably due to competition between transport sites in the plasma membrane between AIB and the natural amino acids

The coronary flow was markedly increased in the pressure overloaded hearts To investigate if the increased coronary flow *per se* stimulated the uptake of AIB and

cycloleucine and if a stimulation of amino acid transport could be demonstrated after a period of increased pressure work load, hearts were perfused with and without pressure overload for 60 min without amino acids in the perfusion medium (Table I). After the initial perfusion period of 60 min AIB ^3H and cycloleucine ^{14}C were added to the perfusion medium and the hearts were perfused for a subsequent period of 30 min at control conditions. It can be seen from Table I that the distribution ratios for AIB and cycloleucine were both significantly elevated in the hearts which had been perfused with increased afterload during the initial 60 min of perfusion. This observation excludes the possibility that the increased coronary flow *per se* was the major factor for the increased uptake of AIB and cycloleucine and that the increased uptake of AIB and cycloleucine was directly dependent upon the increase in oxygen consumption and the substrate oxidation in the pressure overloaded hearts. To elucidate the role of a continuous synthesis of proteins for the stimulation of the amino acid uptake by increased afterload, cycloheximide (10^{-4} M) was added to the perfusion medium at the start of the perfusion. AIB ^3H and cycloleucine ^{14}C were added after an initial perfusion period of 60 min and the concentrative uptake of the amino acids was studied during the subsequent perfusion period of 30 min. Table I shows that the uptake of AIB and cycloleucine still was elevated during the subsequent perfusion period of 30 min in the hearts which had been perfused for 60 min with increased afterload and in presence of cycloheximide. This strongly suggests that the increased uptake of AIB and cycloleucine in the pressure overloaded heart is not dependent upon a concomitant stimulation of protein synthesis. In a control experiment the magnitude of inhibition of protein synthesis by cycloheximide at a concentration of 10^{-4} M was investigated. In this experiment the hearts were perfused for 60 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) and phenylalanine ^3H without supplementary amino acids. The incorporation was 155 ± 11 and 7 ± 1 DPM/mg protein $\times 10^{-1}$ in absence and presence of the inhibitor, respectively (6 hearts in each group). This gives a 95 % inhibition of the protein synthesis with the concentration of cycloheximide (10^{-4} M) used in the experiment.

Effect of increased volume load (increased preload) on the accumulation of AIB ^3H and cycloleucine ^{14}C

In previous experiments (Hjalmarson and Isaksson 1972 a) it was reported that increased preload did not stimulate the incorporation of phenylalanine ^3H in the perfused working rat heart, although the external cardiac work was increased 3 times and the glucose uptake was elevated approximately 100 %. Table II shows a similar experiment in which the uptake of AIB was not stimulated when the left atrial filling pressure was increased from 2 to 15 cm H_2O in hearts perfused for 60 min. This indicates that the active transport of amino acids is not directly linked to the glucose uptake, which is increased during this condition. The data also suggest that the uptake of amino acids is specifically stimulated by an increase in the afterload and is not correlated to the external work of the heart.

TABLE II Effects of increased left atrial filling pressure on AIB-C uptake of isolated working rat heart

Parameter	Left atrial filling pressure cm H O		
	2	7	15
AIB-H distribution ratio	3.54 ± 0.37	3.52 ± 0.16	3.90 ± 0.18

Hearts were perfused anterogradely (working) for 60 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) at various left atrial filling pressures. Values are means ± S.E. Five hearts in each group were perfused.

Effect of increased afterload on hearts from hypophysectomized rats

It has been demonstrated that hearts from hypophysectomized rats do not hypertrophy in response to aortic constriction and it has been postulated that work induced cardiac hypertrophy is dependent upon the presence of growth hormone and thyroxine (Beznak 1964). In the present study it was found that pressure overload stimulated the uptake of AIB in hearts from hypophysectomized rats perfused for 60 min (Table III). This indicates that increased afterload stimulates the amino acid uptake in the heart also when the hormone balance is markedly altered. It is further seen from the table that the distribution ratio of the utilizable amino acid phenylalanine is significantly decreased by elevated pressure load in hearts from hypophysectomized rats. This is probably due to the fact that the rate of incorporation proceeds at a more rapid rate than the membrane transport of phenylalanine. The distribu

TABLE III Effects of increased pressure work load on incorporation of phenylalanine H and accumulation of phenylalanine H and AIB-H in the isolated working heart from normal and hypophysectomized rats

Parameter	Hypophysectomized		Normal	
	Control	Pressure overload	Control	Pressure overload
Phenylalanine H incorporation DPM/mg protein × 10	168 ± 4 (6)	193 ± 6 (6)[b]	179 ± 8 (4)	249 ± 10 (4)[d]
	p < 0.05		p < 0.05	
Phenylalanine H distribution ratio	1.16 ± 0.07 (6)	1.03 ± 0.09 (6)[f]	1.36 ± 0.04 (4)[g]	1.32 ± 0.02 (4)[h]
	p < 0.05			
AIB-H distribution ratio	1.44 ± 0.21 (7)[i]	2.92 ± 0.28 (6)[j]	4.8 ± 0.36 (5)[k]	7.41 ± 0.46 (5)[l]
	p < 0.05		p < 0.05	

Hearts were perfused anterogradely for 60 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM). All normal amino acids at normal plasma levels were included when the incorporation and accumulation of phenylalanine H was studied. Values are means ± S.E. Number of hearts in each group is indicated in parentheses.
b vs d [illegible] e f vs g h [illegible] i s k [illegible] j [illegible] l [illegible] p < 0.0 [illegible] NS

TABLE IV Mechanical performance of hearts from hypophysectomized and normal rats

Parameter	Hypophysectomized		Normal	
	Control (6)	Pressure overload (6)	Control (6)	Pressure overload (6)
Heart rate beats/min	165±8	171±11	252±16	246±12
Peak systolic pressure mm Hg	82±2	115±11	96± 4	164± 9
Diastolic pressure mm Hg	49±3	88± 6	45± 3	122± 6
Coronary flow ml/g d w × min	55±4	118± 4	76± 6	187± 6

Hearts from normal and hypophysectomized rats were perfused anterogradely (working) for 60 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) and all amino acids at normal plasma levels The pressure overload was accomplished by total constriction of the aortic tube Values are means±S E Number of hearts in each group is indicated in parentheses

tion ratios of AIB and phenylalanine were significantly lower both in the control and overloaded hearts from hypophysectomized rats compared to the corresponding groups of normal rats This indicates that the membrane transport of amino acids is decreased after hypophysectomy

Pressure overload also stimulated the incorporation of phenylalanine into proteins of hearts from hypophysectomized rats perfused for 60 min with Krebs Henseleit bicarbonate buffer containing all amino acids at normal plasma levels (Table III) The incorporation rate of phenylalanine is significantly lower in the pressure overloaded hearts from hypophysectomized rats compared to pressure overloaded hearts from normal rats In a previous investigation (Hjalmarson *et al* 1970) it was demonstrated that a significantly lower incorporation rate of phenylalanine in hearts from hypophysectomized rats compared to normal rats could be demonstrated when the heart protein synthesis was stimulated by addition of palmitate and all supplementary amino acids to the perfusion medium This suggests that a difference in the incorporation rate of phenylalanine can be demonstrated between hearts from normal and hypophysectomized rats when the protein synthesis is accelerated *e g* by overload palmitate or high levels of amino acids Table IV shows that the mechanical performance between hearts from normal and hypophysectomized rats were quite different This difference was especially marked between pressure overloaded hearts from normal and hypophysectomized rats Heart rate peak systolic pressure and coronary flow were decreased in overloaded hearts from hypophysectomized rats compared to hearts from normal rats The decreased pressure development in these heart could explain why the incorporation rate of phenylalanine and uptake of AIB is depressed in pressure overloaded hearts from hypophysectomized rats since it has been shown that the magnitude of the increase in afterload is important for the quantitative stimulatory effect on phenylalanine incorporation (Hjalmarson and Isaksson 1972 a)

Discussion

The rate of amino acid transport appears to be closely correlated to the rate of protein synthesis in skeletal muscles as in other growing tissues (Christensen 1962 Griffiths and Pirt 1967 Riggs 1964) It is for example well known that insulin and growth hormone which promote protein synthesis in several tissues accelerate amino acid uptake while glucocorticoids which promote muscle wasting decrease amino acid transport in skeletal muscle (for ref see Riggs 1964) In skeletal and heart muscle most of the natural L-amino acids are accumulated against a concentration gradient i e the concentration of the free amino acid in the intracellular water is several times that of the plasma or extracellular fluid Anoxia and poisons of oxidative phosphorylation generally decrease the accumulation of amino acids which indicates that the transport is an active one (Christensen 1962) Since active mechanisms are involved it has been suggested that the rate of amino acid transport might be rate limiting in protein synthesis (Christensen 1962)

In the present study increased pressure load stimulated the amino acid transport in the isolated working rat heart A significant effect of pressure overload could be seen with various substrates in the perfusion medium after a perfusion period of only 15 min In a previous report (Hjalmarson and Isaksson 1972 a) it was shown that increased pressure load stimulated the protein synthesis and that protein synthesis still was stimulated during a subsequent perfusion period *in vitro* when heart work was normalized This observation suggests that the stimulatory effect of increased afterload was not directly dependent upon the concomitant increase in coronary flow In the present study the uptake of AIB and cycloleucine was also found to be increased after a preceding period with increased pressure load on the heart (Table I) The accelerated amino acid uptake during this experimental condition further strengthens the hypothesis that the coronary flow was not a major determinant for the observed effects The observation that the amino acid transport and protein synthesis are still elevated after normalization of pressure load on the heart indicates that cellular processes which are accelerated by pressure overload continue to be stimulated for some time

It was found in a previous study (Hjalmarson and Isaksson 1972 a) that an increase in preload did not change the rate of incorporation of phenylalanine into heart protein The present experiments show that also the rate of amino acid uptake was unchanged after such an increase in preload This demonstrates how close the rate of amino acid uptake and the rate of protein synthesis seem to be correlated Glucose uptake and oxygen consumption are accelerated when the preload is increased in the isolated working rat heart (Neely *et al* 1967 b Hjalmarson and Isaksson 1972 a) The obvious difference between changes in glucose transport and changes in rate of amino acid transport in hearts perfused with various preloads indicates that increased pressure load specifically stimulates amino acid uptake

The finding that the amino acid uptake still is accelerated by increased afterload when the protein synthesis is inhibited to 95 % by cycloheximide (Table I) suggests that the increased amino acid uptake is not dependent upon a continuous

synthesis of proteins. This of course does not show that the accelerated amino acid transport triggers the increase in protein synthesis. It is possible that amino acid transport and protein synthesis are stimulated through a common mechanism simultaneously. The decreased distribution ratios of AIB and cycloleucine in presence of cycloheximide compared to hearts perfused without this inhibitor are compatible with the theory that protein(s) participate in the active trans-membrane transport of amino acids in animal cells (Adamson *et al.* 1966, Kostyo and Redmond 1966).

Several studies have shown that the weight of the heart decreases after hypophysectomy (McQueen-Williams and Thompson 1940, Hajdu and Beznak 1945). The accelerated catabolism in the hearts after hypophysectomy can be explained in principally two ways: (i) absence of anabolic hormones such as growth hormone and thyroxine, (ii) decreased peripheral oxygen consumption with concomitant decreased physiological demand on the heart, (iii) or both. It has also been proposed that growth hormone and thyroxine are required for development of cardiac enlargement after aortic constriction in hypophysectomized rats (Beznak (1964). Other studies have shown that cardiac hypertrophy can be induced in hypophysectomized animals if the pressure load on the heart is adequate (Lipana and Fanburg 1970, Morkin et al. 1968). The latter studies are also supported by the observation that work induced hypertrophy of skeletal muscles is not dependent upon the presence of an intact pituitary gland (Goldberg 1967). Although not quite comparable to the *in vivo* situation, the results of the present study suggest that protein synthesis and amino acid transport can be stimulated by increased pressure load *in vitro* in hearts from hypophysectomized rats. The decrease in the distribution ratio of AIB and phenylalanine in hearts from hypophysectomized rats suggests that the membrane transport of amino acids is decreased in the heart in the absence of an intact pituitary gland. In a previous investigation only a small decrease in the rate of AIB uptake was found after hypophysectomy (Hjalmarson *et al.* 1969). The decrease was so small that it could be explained by the fact that the hypophysectomized rats were compared with normal rats 2 weeks younger. It is well known that AIB uptake is decreased in heart and skeletal muscle when rats grow older (Riggs and Walker 1958, Hjalmarson 1968, Hjalmarson *et al.* 1969). The decrease in the present study was, however, so marked that it can not be explained by a difference in age. The discrepancy between the two studies might be explained by the fact that the per cent decrease in heart weight after hypophysectomy is more pronounced in older than in younger rats (unpublished observations).

In the present study the membrane transport of amino acids was studied using the two non-utilizable amino acids AIB and cycloleucine. Whether these amino acids are representative for normal amino acids can be questioned. Serious criticism has been directed against AIB since it has been shown for several tissues that the intracellular concentration of this model amino acid continues to increase for very long period in a way which contrasts markedly to the behaviour of most normal amino acids. The behaviour of AIB can, however, easily be explained by the fact that 1. it is not metabolized in the cell and not incorporated into protein, 2. the rate

of active cellular uptake dominates over the rate of active transport out of the cell and 3 exchange diffusion is very slow Cycloleucine shows quite another behaviour more similar to that of many normal amino acids since this amino acid analogue has a more rapid transport out of the cells and in addition a more pronounced exchange diffusion It is therefore a clear advantage to use both AIB and cycloleucine when studying amino acid transport There are several reports favouring the interpretation that AIB and cycloleucine are transported into the muscle cells in a manner similar to several natural amino acids (e g Akedo and Christensen 1962 Christensen and Jones 1962 Segal *et al* 1966) Of relevance when discussing amino acid transport are the theories of Oxender and Christensen (1963) that there exist at least two different transport systems for neutral amino acids the L and the A system and it has been demonstrated that AIB is competing with amino acids transported via the A system while cycloleucine is competing with amino acids transported via both the A and the L system The finding in the present study that increased pressure load accelerated both the uptake of AIB and the steady state distribution of cycloleucine indicates therefore very strongly that increased pressure load has an effect also on the membrane transport of normal amino acids

It has already been mentioned in a previous investigation (Hjalmarson and Isakson 1972 a) that increased afterload increased the incorporation rate of phenylalanine in the perfused heart The finding in the present study that amino acid transport is increased under similar conditions raises the question how these processes are connected What seems to be required for a proper study of the relationship between amino acid transport and protein synthesis in muscle is a chemical determination of the exact amount of each natural amino acid in the intracellular water in circumstances under which the protein synthesis is accelerated and deaccelerated Such studies have been carried out in the perfused rat heart (Scarff and Wool 1966 Morgan *et al* 1971 a Jefferson *et al* 1971) In these studies no consistent correlation was found between the intracellular amino acid concentrations and the rate of protein synthesis and it was therefore concluded that changes in the rate of amino acid transport probably could not have a physiological significance in relation to the rate of protein synthesis This conclusion is however valid only if a homogeneous intracellular pool of amino acids exists and if this pool is on the route of protein synthesis There exists some evidence that this could be the case for some amino acids In the isolated rat heart Morgan *et al* have shown that lysine glycine and phenylalanine equilibrate with a homogeneous intracellular pool of amino acids before incorporation into heart protein (Morgan *et al* 1971 a) On the other hand from studies on the extensor digitorum muscle in the rat Hider *et al* (1969 1971 a b) presented evidence indicating that amino acids entered cell protein direct from a small extracellular pool without prior equilibration with the intracellular amino acid pools Instead they proposed that the biological role of the amino acid pool was to act as an reservoir e g during starvation Other studies favouring the existence of a functional heterogeneous intracellular amino acid pool have been put forward (Reiss and Kipnis 1959 Kipnis *et al* 1961 Kostyo 19

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Catecholamines and Cholinesterases in the Paracervical (Frankenhauser) Ganglion of Normal and Pregnant Rats

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Abstract

Kanerva L, R Lietzen and H Teräväinen *Catecholamines and cholinesterases in the paracervical (Frankenhauser) ganglion of normal and pregnant rats* Acta physiol scand 1972 *86* 271—277

Chatecholamines and cholinesterases were studied histochemically in the uterine paracervical ganglion of normal and pregnant rats and after both sympathectomy and sacral para sympathectomy About one fifth of the ganglion cells revealed intense acetylcholinesterase (AChE) activity The cells were large and devoid of catecholamines and received AChE con taining nerves — About one third of the ganglion cells contained weak to moderate yellow green catecholamine fluorescence They were presumed to be adrenergic although they also exhibited moderate to weak AChE activity These postganglionic neurons represent short adrenergic neurons The rest of the ganglion cells with moderate to weak AChE activity were presumed to be cholinergic Increased ganglion cell fluorescence was observed after 2 weeks of pregnancy There was no change in the cholinesterase activity of the neurons — Division of the sympathetic or the sacral parasympathetic nerves did not cause changes in the intensity of the histochemical reactions The small intensely fluorescent cells (SIF cells) did not show AChE activity Other cholinesterases although present in the cytoplasm of the ganglion cells and SIF cells were mainly localized in the glial cells

The presence of the paracervical ganglion on both sides of the uterus approximately at the utero vaginal junction was established long ago (Frankenhauser 1867 *cf* Krantz 1959) However only a few histochemical studies have been made on the paracervical ganglion The first report on the cholinergic part of the neurons of the paracervical ganglion was made by Makela and Gronroos (1959) They distinguished both acetylcholinesterase (AChE) containing neurones and neurones devoid of AChE activity within the ganglion The paracervical ganglion of the uterus also con tains both catecholamine containing and non adrenergic nerve cells in addition to many small intensely fluorescent cells (SIF cells) (Sjoberg 1967 Kanerva 1971 Hervonen *et al* 1972 Kanerva and Teravainen 1972)

The present work was planned as a more detailed histochemical study of the different neuronal cell types of the paracervical ganglion of the adult rat The aim was to clarify their AChE or catecholamine content and the effect of pregnancy sympathectomy and parasympathectomy

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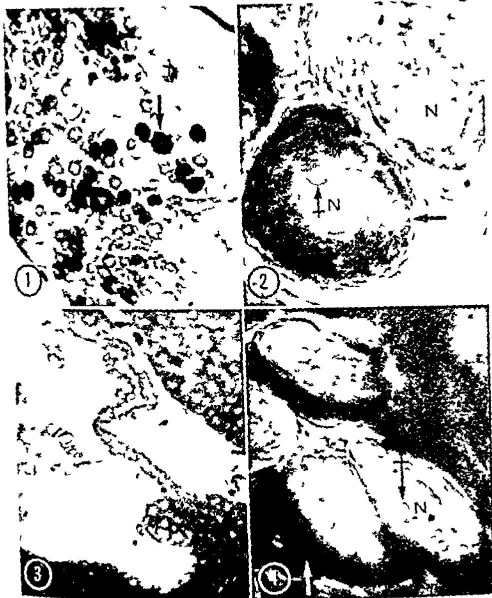
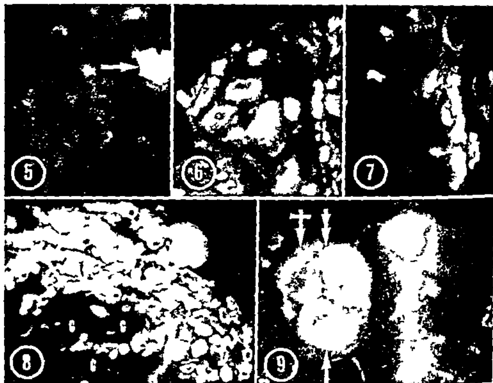


Fig 1 ACrE activity of the paracervical ganglion About one fifth of the ganglion cells contain strong AChE activity (arrow) These cells are larger than the cells with moderate to weak AChE activity (× 150)

Fig 2 The most intense AChE activity in the ganglion cells is situated close to the cell membrane (arrow) where the synapses are located Nucleus N nucleolus crossed arrow (× 2000)

Figs. 3—4 Ns ChE activity of the paracervical ganglion The activity is localized mainly in the interstitial tissue (arrow) between the neural cells Nucleus N nucleolus crossed arrow (Fig 3 × 150 Fig 4 × 1500)



Figs 5—7 The effect of pregnancy on the formaldehyde induced fluorescence (FIF) of the paracervical ganglion (× 200) Fig 5 Estrous rat weak FIF of the ganglion cells a small group of SIF-cells (arrow) Fig 6 2 weeks of pregnancy an increased FIF of ganglion cells. Fig 7 3 weeks of pregnancy The FIF of the ganglion cells corresponds to the FIF of non-pregnant animals

Fig 8 The paracervical ganglion of the rat contains large amounts of SIF cells The sites of the ganglion cells (G) appear black because of underexposure to avoid overexposure of the SIF cells (× 400)

Fig 9 Fluorescent nerve fibres (double arrow) were rarely encountered close to fluorescent (arrow) or non fluorescent (crossed arrow) ganglion cells (× 500)

The most intense non specific cholinesterase (ns ChE) activity was confined to the structures between the ganglion and the SIF cells (Fig 3 4) The electron microscope showed this space to be occupied by the satellite cells These cells also surround the nerve fibres arriving in the proximity of the nerve cells (Kanerva and Teräväinen 1972) There appeared to be weak ns ChE activity in the cytoplasmic components of the ganglion cells and SIF cells since the region of their nucleus and nucleolus like the nucleus of the satellite cell were visualized as pale areas Activity due to ns ChE was also seen along the nerve bundles

No definite difference in AChE or ns ChE activity could be detected between the diestrus and estrus rat or after 1 2 or 3 weeks of pregnancy

Aromatic monoamines About one third of the ganglion cells emitted weak to moderate greenish catecholamine fluorescence the rest being nonfluorescent The fluorescent and nonfluorescent ganglion cell were unevenly distributed within the ganglion their proportional amount varying in different sections These fluorescent cells were smaller than the nonfluorescent ganglion cells Fluorescing nerve fibres were only occasionally encountered close to ganglion cells (Fig 9)

Round to slightly elongated small cells 5 to 10 μ in diameter with an intense yellowish fluorescence (SIF-cells) (Fig 8) were een among the fluorescent and non fluorescent ganglion cells These cells were often arranged in clusters containing as many as 50 cells in a single section The nonfluorescent nucleus was surrounded by a narrow ring of intensely fluorescent cytoplasm sometimes extending to a short process 5 to 10 μ in length These cells were also seen outside the ganglion in the connective tissue (Kanerva 1971)

The fluorescence intensity of ganglion cells was much the same during the estrus (Fig 5) diestrus and after one week of pregnancy but was somewhat stronger after 2 weeks of pregnancy (Fig 6) After 3 weeks of pregnancy the intensity of fluorescence had again decreased (Fig 7) to the level of non pregnant rats The amount of non fluorescent cells remained the same at all the stages studied The SIF-cells exhibited a strong fluorescence during the whole period investigated and no marked changes in their number or fluorescence intensity could be observed

Operative procedures Lumbar sympathectomy and sacral parasympathectomy caused no clear changes in the catecholamine fluorescence or in the cholinesterases of the different cells The same applied to the content of AChE-containing nerve fibres within the paracervical ganglion

Discussion

It is generally accepted that AChE is a good marker of cholinergic neurons (for ref see Eränkö 1967 b) One can thus assume that the large ganglion cell which comprise about one fifth of the neurones of the paracervical ganglion and contain intense cytoplasmic AChE activity are cholinergic since ganglion cell of the same size were devoid of catecholamine fluoresence It is somewhat more unsafe to draw conclusions about the cholinergicity of the rest of the AChE containing ganglion cells possessing moderate to weak AChE activity Because only about one third of the ganglion cells are presumably adrenergic since they exhibit moderate to weak greenish formaldehyde induced fluorescence it seems that a large part of the neurons containing moderate AChE are cholinergic Apparently all the ganglion cells of the rat paracervical ganglion contained AChE activity at least to some degree It is therefore probable that the aminergic neurons also contain AChE activity as Eranko and Harkonen (1964) have shown in the superior cervical ganglion

The cell bodies of the adrenergic neurons of the paracervical ganglion of the rat uterus are close to the target organs (Kanerva *et al* 1972) They can thus be considered short adrenergic neurons (Owman and Sjostrand 1965) The hort

adrenergic neurons innervating the female genital organs (Sjoberg 1967) have been found to differ from the long adrenergic neurons in many respects One of these differences is the increase in number and fluorescence intensity of the short adrenergic nerves in the uterus during the first half of pregnancy (Sjoberg 1967) This has been assumed to be due to estrogen and specific for female animals (Owman *et al* 1970) It seems to be not only axons but also the cell bodies of the short adrenergic neurons which might be affected by hormonal changes in the animal Kennedy (1929) reported that estrogen caused the Nissl substance of the paracervical ganglion cells to be chromophilic and conglomerated It the absence of estrogen however the ganglion cells became chromophobic and agglomerated

The SIF cells in the superior cervical ganglion were first described by Eränko and Harkonen (1963) and have since been studied by both fluorescence and electron microscopy in many autonomic ganglia (*cf* Jacobowitz 1970 Eranko and Eranko 1971 Kanerva and Teravainen 1972) The paracervical ganglion also contains many SIF cells which are located close to the blood capillaries These might secrete catecholamines under hormonal control (see Kanerva and Teravainen 1972) Since the beginning of this century cells giving a positive chromaffin reaction have been found in many autonomic ganglia with classical methods (Kohn 1903, *cf* Lempinen 1964) Blotevogel (1925 1927) and Hofbauer (1950) noticed a significant increase in the number of chromaffin cells in the paracervical ganglion during estrus and at the end of pregnancy We were unable to confirm this however by using the formaldehyde induced fluorescence method The chromaffin reaction demonstrate cells containin a high concentration of catecholamines (see Coupland 1965 Eranko and Eranko 1971) The fluorescence method on the other hand is extremely sensitive and even very small amounts of catecholamines can be detected (*cf* Eranko 1967 b) The increase in chromaffin cells in the paracervical ganglion during estrus and pregnancy (Blotevogel 1925 1927) might, thus merely have been due to the increase in the catecholamine concentration of the SIF cells

Preganglionic division of the sympathetic nerves or the sacral parasympathectomy was not observed to cause a clear reduction in the AChE containing nerves within the ganglion This is in contrast to the preganglionic denervation of the superior cervical ganglion (Harkonen 1964) or the ciliary ganglion (Huikuri 1966) and the electron microscopical findings of Mustonen and Teravainen (1971) in the paracervical ganglion Based on these contraversial observations it is possible that quantitative and qualitative determinations were difficult to perform It is also feasible that total denervations were not accomplished due to the many ganglia and nerves in the pelvis nerve plexus that are difficult to see

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Differential Effect of para-Chlorophenylalanine on the Two Slow Wave Sleep Stages in the Cat

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Abstract

URSIN R *Differential effect of PARA chlorophenylalanine on the two slow wave sleep stages in the cat* Acta physiol scand 1972 86 278—285

The effect of an injection of 200 mg/kg *para* chlorophenylalanine (PCPA) on sleep was studied in 6 cats 48—72 h after the injection Each cat was also given a control injection of the vehicle (BRIJ 35) 3 weeks before or after the experimental dose in a balanced order The main effect of PCPA was a reduction of the deep slow wave sleep (DSWS) with little effect upon the light slow wave sleep (LSWS) or rapid eye movement (REM) sleep Both the number and the length of the DSWS episodes were reduced For LSWS only the length of the episodes was reduced The number and the length of the REM sleep episodes were not changed When the DSWS was reduced to 10 % or less of control values there was a tendency to a decrease of LSWS and REM sleep also

It is well known that *para* chlorophenylalanine (PCPA) administration to cats reduces their sleep PCPA inhibits tryptophan hydroxylase and consequently the formation of 5 hydroxytryptamine (5HT serotonin) is reduced PCPA also inhibits the phenylalanine hydroxylase which is necessary for hydroxylation of phenylalanine to tyrosine However brain catecholamines are only slightly lowered (Koe and Weissman 1968) The decrease of brain 5HT is believed to be the important factor for the sleep reduction although an effect via altered catecholamine metabolism is not entirely ruled out (Koella *et al* 1968 Pujol *et al* 1971) It is uncertain how low brain 5HT affects sleep Jouvet (1969) has suggested that serotonin is essential for the mechanism underlying slow wave sleep (SWS) Dement (1969) has proposed that low brain 5HT disturbs the sleep pattern by releasing ponto geniculate occipital activity during SWS and the waking state This activity normally occurs only during rapid eye movement (REM) sleep and the immediately preceding SWS It is also possible that somatic or vegetative effects of lowered brain 5HT indirectly affect sleep

The *effect of PCPA is evident even after a single dose* the maximal effect starting 24 to 48 h after the injection The reduction of sleep is almost complete when

the dose is above 300 mg/kg (Delorme *et al* 1966 Pujol *et al* 1971) With lower doses both SWS and REM sleep have been reported to decrease to a varying degree (Koella *et al* 1968)

The aim of the present paper was to study more closely the effects of PCPA on the organization of sleep in particular the effects on the two stages of slow wave sleep (Ursin 1968) The uncertainty as to what sleep mechanism PCPA interferes with may derive in part from an imprecise definition of SWS In all previous PCPA studies in the cat SWS has been looked upon as an entity However the differentiation of SWS into two stages has been shown to be functionally meaningful (Ursin 1968 1970 1971) Two stages of SWS have been quantified light slow wave sleep (LSWS) and deep slow wave sleep (DSWS) These two stages seem to serve different functions In the analysis of the sequential organization of sleep LSWS is related to wakefulness and DSWS to REM sleep LSWS usually initiates sleep and is followed by DSWS which in turn precedes REM sleep There are also differences in the quantitative relationships between these two stages and REM sleep There is a positive correlation between quantities of DSWS and quantities of REM sleep while no such relationship exists for LSWS and REM sleep It may aid understanding of the PCPA effects to know whether both slow wave sleep stages are affected by this drug and whether the normal relationships between the sleep stages are intact

Materials and methods

6 cats were used in the main experiment 5 male and 1 female The implantation procedure has been described in detail elsewhere (Ursin 1968) Stainless steel needle electrodes for EEG recordings were implanted bilaterally over the frontal and occipital regions (Horsley and Clarke coordinates were A 18—20 and L 10 P 5 and L 8—10 respectively) Neck EMG and eye movements were also recorded

A Lehigh Valley Electronics Chamber Model 1488 (87 cm × 73 cm × 90 cm) was used as recording chamber The cat was connected to the recording equipment via a light and flexible cable and a slip ring connector permitting free movement The cat could be observed through a one way mirror covering the whole door of the chamber A fan was on throughout the experimental session The temperature in the recording chamber was not thermostatically controlled but it usually stayed around 24—25 C

The cats were habituated to the recording chamber by spending at least one 24 h period (approximately 0900 h—0900 h) in the chamber for 8 h of which they were connected to the cable In addition all cats were subjected to one 24 h recording without injection before the experiment took place

In the experiment proper the sleep of each cat was recorded for one 24 h period starting 48 h after PCPA injection and for one 24 h period starting 48 h after a control injection 3 cats chosen at random had the control experiment first (cats no 1 3 and 6) the other 3 had the PCPA experiment first PCPA was injected intraperitoneally 00 mg/kg suspended in a solution of BRIJ35 (1/2 % in 0 9 % sterile NaCl) The injected volume was 5 ml/kg The control injection consisted of a similar quantity of BRIJ35 solution alone At least 3 weeks passed between the experiments No clinical signs of peritonitis were present at the time of recording

The recordings started around 0900 h The cat was given his daily ration of food and milk at 1900 h or whenever he woke up thereafter This was the usual feeding time in the living quarters

Recordings were done with alternating speeds of 1 5 mm/s and 30 mm/s one page (30 cm) each. Behavioral notes were taken each time the speed was changed to low The scoring criteria both for the high speed and slow speed recording has been described in detail earlier (Ursin 1968) In short the following stages were identified (high speed criteria) *Awake* including drowsiness *Light slow wave sleep* (LSWS) characterized by 12—14 c/s high voltage sleep spindles and some 1—4 c/s high voltage slow waves on a low voltage background *Deep*

TABLE I Total sleep, LSWS, DSWS and REM sleep given in per cent of recording time for control recordings (C) and recordings 48 to 72 h after injection of 200 mg/kg PCPA

Cat no	Total sleep		LSWS		DSWS		REM S		REM S/DSWS	
	C	PCPA	C	PCPA	C	PCPA	C	PCPA	C	PCPA
1	39.3	36.2	15.1	12.9	16.0	14.1	8.2	9.2	0.51	0.65
2	44.2	30.6	13.3	14.7	19.8	6.0	11.1	10.0	0.56	1.67
3	42.4	7.8	17.6	4.7	19.9	0.6	4.9	2.6	0.25	4.33
4	49.8	0.4	11.9	0.4	24.9	0.0	13.0	0.0	0.52	–
5	41.6	25.9	11.4	9.5	24.0	8.8	6.3	7.6	0.26	0.86
6	43.1	14.2	12.4	8.9	20.3	2.0	10.3	3.3	0.51	1.65
Mean	43.4	19.2	13.6	8.5	20.8	5.3	8.9	5.5	0.44	1.83
S.D.	3.5	13.9	2.3	4.7	3.2	5.5	3.1	4.0	0.14	1.47
Sign. level*	P < 0.02		Not sign.		P < 0.01		Not sign.		Not sign.	

* Sandler's A statistics for correlated samples, two tailed

slow wave sleep (DSWS), when more than 50% of a page (10 s) consisted of slow waves and spindles. *REM sleep*: low voltage fast waves in the EEG and lack of muscle tonus in the EMG, bursts of rapid eye movements. By cross checking the 2 speeds a special set of criteria has been developed for reading the slow speed records. Interobserver agreement is satisfactory with these sets of criteria (Ursin 1968).

All records were read by the same experimenter. The records were read in 1 min epochs by scoring clock time interval. When more than one stage was present in one epoch, the stage that occupied most of the epoch was scored. Clock time and diagnosis were then punched on cards for computer processing.

The results after control injections were compared with the results after the PCPA injection in each cat. Sandler's A statistics was used in the statistical evaluation of the differences (Runyon and Haber 1968). This test is based on a t test for correlated samples. The FORTRAN subroutine SRANK was used in the sleep cycle correlation analysis. For the other correlated data the Pearson product moment correlation coefficient was determined (Runyon and Haber 1968).

Results

Behavior

Some of the cats were more restless and active under PCPA administration than in the control condition. Apart from this, their general behavior was not changed; they ate, drank, groomed and used the sawdust box as usual.

A striking difference appeared between experimental and control recordings in the EEG pattern of quiet resting cats. Under the control condition they often showed the drowsiness pattern (see Ursin 1968). In the experimental recordings the EEG was remarkably desynchronized when the cats were quiet, and the drowsiness pattern was seen more seldom than in the control recordings.

Total sleep time and composition of sleep

Total sleep time was decreased in all cats after PCPA injection. The decrease was statistically significant ($P < 0.02$). The sleep stage most consistently reduced was DSWS (Table I). It was lower in all cats after PCPA injection ($P < 0.01$). There

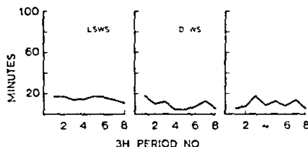


Fig 1 Minutes of LSWS DSWS and REM sleep per 3 h period 48—72 h after injection of 200 mg/kg PCPA (continuous line) and 48—72 h after a control injection (broken line)

was no significant change in LSWS or in REM sleep when the 6 cats were regarded as a group However the LSWS and REM sleep stages were reduced in the 3 cats where DSWS was lowest (10 per cent or less of control values) In the other 3 cats there was no change There was thus a tendency to a stepwise effect of the PCPA injection In cat no 1 2 and 5 there was an effect on DSWS only In cat no 3 4 and 6 all three sleep stages were reduced

The relative proportion of DSWS (DSWS per cent of total sleep) was significantly reduced ($P < 0.01$ two-tailed) The relative proportion of LSWS increased but this did not reach significance ($P < 0.10$) REM sleep per cent of total sleep was variable after PCPA injection it increased in 4 cats and decreased in 2 in the whole group it was not significantly changed

Total quantities of DSWS and REM sleep were positively and significantly correlated in the experimental records ($r = 0.84$ $P < 0.025$) This correlation coefficient was shown to vary between 0 58—0 68 in earlier materials (Ursin 1968 1971) In the present injected control records it was considerably lower $r = 0.25$ This may indicate an effect of the control injections on the relation between DSWS and REM sleep but this effect was opposite to the effect of PCPA injection

The ratio REM sleep/DSWS was increased in all cats (Table I) but the increase was not significant

Sleep stage variation throughout the 24 h recording period

The mean quantity of each sleep stage per 3 h period was determined (Fig 1) DSWS was significantly reduced in periods no 2 3 and 5 ($P < 0.01$) and in period no 6 ($P < 0.05$) The effect of PCPA on DSWS thus was present throughout most of the recording period Although the values for LSWS and REM sleep were generally lower after PCPA injection than after the control injection they were not significantly different from control values

There was a significant positive correlation between the mean hourly quantities of DSWS and REM sleep in the experimental recordings ($r = 0.58$ $P < 0.005$) In earlier normative materials the correlation coefficients were 0 63 and 0 72 (Ursin 1970 1971) In the present injected controls the correlation coefficient was 0 28 and not significant

TABLE II Number of episodes and episode lengths Means and significance level of differences (Sandler s 1 statistics two tailed)

	Control	PCPA	Sign level
Episode number			
Awake	90	77	Not sign
LSWS	112	83	Not sign
DSWS	78	34	P<0 0?
REM sleep	24	19	Not s gn
Episode length (min)			
Awake	9 12	49 81	Not sign
LSWS	1 75	1 41	P<0 05
DSWS	3 88	1 88	P<0 02
REM sleep	5 44	5 ?4	Not sign

Episode number and length

The number of DSWS episodes was significantly reduced (P < 0 05 Table II) The number of LSWS and REM sleep episodes were not significantly changed Both LSWS and DSWS episodes were significantly shorter in the PCPA recordings (P < 0 05 and P < 0 02) while the REM sleep episode length was unchanged There was no significant change in the number or length of waking episodes

Sleep stage alternation

Under PCPA administration there were some disturbances in the stage alternation described previously (Ursin 1970) The waking state was succeeded by LSWS as 1 From LSWS however there was a significantly higher tendency to go back into the waking state and a correspondingly lower tendency to go into DSWS sleep (Table III) When the cats did enter DSWS the progression into REM sleep was as usual REM sleep was preceded by DSWS significantly less often than in the control condition There was more REM sleep preceded by LSWS and more REM sleep preceded by the awake state than normal although this did not reach significance

The sleep cycles

The sleep cycle was defined as the time from the start of one REM sleep episode to the start of the next REM sleep episode The sleep cycles that had less than 4 min inter REM sleep or more than 10 min of the awake state were excluded (Ursin 1970 1971) Only 4 of the cats had sleep cycles that were accepted In all these 4 cats the sleep cycles were shorter under the experimental condition than under control conditions but the difference did not reach significance (P < 0 10)

Intra cycle correlations

In earlier normative data it was shown that when 48 h recordings are considered there is a significant positive correlation between the length of a REM sleep episode and the quantity of DSWS in the succeeding inter REM period (Ursin 1970) In the present 24 h recordings the number of cycles obtained for analysis for each cat

Table III Sleep stage alternation Means for 6 cats

Preceding stage				Control stage	Succeeding stage			
Awake	LSWS	DSWS	REM S		Awake	LSWS	DSWS*	REM S
	43	36	20	Awake (n = 90)		91	9	0
12		23	5	LSWS (n = 112)	34		62	4
11	89		0	DSWS (n = 18)	42	34		25
0	18	82		REM S (n = 24)	78	22	0	
				PCPA				
	61	16	23	Awake (n = 16)		94	5	1
8		11	2	LSWS (n = 82)	57		36*	7
12	88		0	DSWS (n = 34)	36	28		36
3	3	65		REM S (n = 19)	91	8	1	

P<0.05 (Significance level of differences Sandler's A statistics two-tailed)
* P<0.01

was much lower than in the 48 h recordings Although the correlation coefficients did not differ to any great extent from those previously published they did not reach significance There was no difference between the correlation coefficients under the experimental and the control conditions

Discussion

A moderate dose of PCPA seemed to have a quite specific effect on cat sleep DSWS was significantly reduced 48—72 h after an injection of 200 mg/kg PCPA Both the number and the length of the DSWS episodes were reduced There were also slight changes in LSWS and REM sleep but these were not consistent in all cats and were not significant in the group as a whole A clear reduction of LSWS and REM sleep was only observed in the 3 cats where DSWS was reduced to 10 per cent or less of control values The present data thus suggest that disruption of DSWS is the primary effect on cat sleep of a moderate PCPA dose The finding is in agreement with observations by Koella *et al* (1968) and Karadzic (1968) that the slow waves disappear from the EEG in PCPA treated cats

The lack of any significant change in the total quantity of REM sleep differs from the findings of other researchers (Delorme *et al* 1966 Koella *et al* 1968 Pujol *et al* 1971) This discrepancy may at least in part be due to different dose levels At high doses (300—500 mg/kg) Delorme *et al* (1966) and Pujol *et al* (1971) reported a complete loss of both SWS and REM sleep At low doses (50—100 mg/kg) Koella *et al* (1968) found that the REM sleep was consistently reduced parallel to the SWS With the same dose level as used in the present experiment (150—200 mg/kg) they found a relative increase of REM sleep in some cats but only when total sleep was very low In the present material REM sleep reduction depended on DSWS reduction In the cats where DSWS was not markedly reduced REM sleep quantity was virtually unchanged

TABLE II Number of episodes and episode lengths Means and significance level of differences (Sandler's A statistics two tailed)

	Control	PCPA	Sign level
Episode number			
Awake	90	77	Not sign
LSWS	112	83	Not sign
DSWS	78	34	P<0.05
REM sleep	24	19	Not sign
Episode length (min)			
Awake	9.12	49.81	Not sign
LSWS	1.75	1.41	P<0.05
DSWS	3.88	1.88	P<0.02
REM sleep	5.44	[illegible].24	Not sign

Episode number and length

The number of DSWS episodes was significantly reduced ($P < 0.05$ Table II) The number of LSWS and REM sleep episodes were not significantly changed. Both LSWS and DSWS episodes were significantly shorter in the PCPA recordings ($P < 0.05$ and $P < 0.02$) while the REM sleep episode length was unchanged There was no significant change in the number or length of waking episodes

Sleep stage alternation

Under PCPA administration there were some disturbances in the stage alternation described previously (Ursin 1970) The waking state was succeeded by LSWS as usual From LSWS however there was a significantly higher tendency to go back into the waking state and a correspondingly lower tendency to go into DSWS sleep (Table III) When the cats did enter DSWS the progression into REM sleep was as usual REM sleep was preceded by DSWS significantly less often than in the control condition There was more REM sleep preceded by LSWS and more REM sleep preceded by the awake state than normal although this did not reach significance

The sleep cycles

The sleep cycle was defined as the time from the start of one REM sleep episode to the start of the next REM sleep episode The sleep cycles that had less than 4 min inter REM sleep or more than 10 min of the awake state were excluded (Ursin 1970 1971) Only 4 of the cats had sleep cycles that were accepted In all these 4 cats the sleep cycles were shorter under the experimental condition than under control conditions but the difference did not reach significance ($P < 0.10$)

Intra cycle correlations

In earlier normative data it was shown that when 48 h recordings are considered there is a significant positive correlation between the length of a REM sleep episode and the quantity of DSWS in the succeeding inter REM period (Ursin 1970) In the present 24 h recordings the number of cycles obtained for analysis for each cat

TABLE III Sleep stage alternation Means for 6 cats

Preceding stage				Control stage	Succeeding stage			
Awake	LSWS	DSWS	REM S		Wake	LSWS	DSWS	REM S
	43	36	20	Awake (n = 90)		91	9	0
72		23	5	LSWS (n = 112)	34		62	4
11	89		0	DSWS (n = 78	42	34		25
0	18	82		REM S (n = 24)	78	22	0	
				PCPA				
	61	16	23	Awake (n = 6)		94	5	1
87 *		11	2	LSWS (n = 82)	57 *		36 *	7
12	88		0	DSWS (n = 34)	36	28		36
3	32	65		REM S (n = 19)	91	8	1	

P<0 05 (Significance level of differences Sandler's t statistics two-tailed)
P<0 01

was much lower than in the 48 h recordings Although the correlation coefficients did not differ to any great extent from those previously published they did not reach significance There was no difference between the correlation coefficients under the experimental and the control conditions

Discussion

A moderate dose of PCPA seemed to have a quite specific effect on cat sleep DSWS was significantly reduced 48—72 h after an injection of 200 mg/kg PCPA Both the number and the length of the DSWS episodes were reduced There were also slight changes in LSWS and REM sleep but these were not consistent in all cats and were not significant in the group as a whole A clear reduction of LSWS and REM sleep was only observed in the 3 cats where DSWS was reduced to 10 per cent or less of control values The present data thus suggest that disruption of DSWS is the primary effect on cat sleep of a moderate PCPA dose The finding is in agreement with observations by Koella *et al* (1968) and Karadzic (1968) that the slow waves disappear from the EEG in PCPA treated cats

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The data from monkeys (Weitzman *et al* 1968) are similar to those of the present study. A single dose of 330—1000 mg/kg PCPA did not change the time the monkeys spent in REM sleep, while the time spent in other sleep stages decreased. The deeper SWS stages were reduced to a greater degree than the lighter SWS stages.

In previous studies on the quantitative relationships between the sleep stages in the cat, two factors of the DSWS/REM sleep relationship have been suggested (Ursin 1968, 1970). A "triggering" factor, possibly neural, was postulated to account for the relatively rapid one-way transition from DSWS to REM sleep. A "duration control" factor, possibly humoral, was postulated to explain the quantitative relationships between DSWS and REM sleep. Neither of these factors was affected by moderate sleep deprivation (Ursin 1971). Both factors were present following PCPA treatment. The "triggering" factor seemed to be intact. When the PCPA treated cats were in DSWS they proceeded to REM sleep as normals. This "triggering" factor may account for the finding that REM sleep was undisturbed as long as there was a minimum of 10 per cent of DSWS left. Still, more REM sleep periods were preceded by LSWS in the PCPA treated cats than in the controls. This finding may indicate that PCPA facilitated the triggering of REM sleep from other states than DSWS.

The "duration control" factor was also present in the PCPA treated cat. The total quantity of DSWS was positively correlated with the total quantity of REM sleep both in the 24 h records and within each hour of the records. For the 24 h record the correlation coefficient was even higher than before (Ursin 1968, 1971). However, the ratio REM sleep/DSWS was increased. For this factor, then, the PCPA treatment seems to have changed the relative proportion of the two stages, but the positive correlation between them remains.

The present data, therefore, support the idea of a direct PCPA effect on sleep mechanisms. The data support Jouvet's (1969) suggestion that PCPA mainly affects SWS mechanisms. However, the effect seems to be even more specific than that. When the dissociation between LSWS and DSWS is taken into consideration, it becomes evident that the effect of a moderate dose of PCPA is primarily on the deep part of the SWS. PCPA thus accentuates the functional dissociation between LSWS and DSWS, and the present data seem to indicate that different mechanisms underlie these two SWS stages. However, it cannot be excluded that the effect of PCPA described here is caused by a more general neurophysiological effect on slow waves, since the LSWS-DSWS dissociation is based mainly on the quantity of slow waves present in the EEG.

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Fast and Slow Units in Extrinsic Eye Muscles of Cat

By

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Although some minor controversies still remain, the existence of fast, twitch fibres and slow, tonic fibres in some of the extrinsic eye muscles seems fairly well established (Hess and Pilar 1963, Bach-y-Rita 1970, Peachey 1970). However it has still to be demonstrated that motor units in these muscles behave electrically and mechanically in the same way as the muscle fibres.

The present preliminary report describes some results obtained in a study of motor units in the inferior oblique muscle of the cat. The muscle was attached to a very sensitive myograph for isometric muscle tension recording. Isolation of single motor units was facilitated by extensive cutting of nerve branches entering the muscle. The activation of a single motor unit was assessed from recordings of EMG and muscle tension in response to single or repetitive stimulation of the intra-orbital part of the muscle nerve.

Fig. 1 shows typical responses of two different types of motor unit with electrical characteristics suggestive of fast and slow muscle fibres respectively. In the fast unit biphasic action potentials were recorded (Fig. 1 A). At frequencies of repetitive stimulation above 200—300/s action potentials started to drop out. In the slow unit an electrical response was obtained that resembled small nerve junctional potentials (Kuffler and Vaughan Williams 1953), summating at frequencies above 50/s (*Fig. 1 B*).

The units differed markedly in their mechanical responses. The fast unit had a short lasting twitch response with a contraction time, measured from start of EMG, of 6 ms (Fig. 1 A), and the frequency of stimulation to reach fully fused tension output was 300/s. The slow unit showed no tension change to single shock stimulation and its fusion frequency could not be determined. The units also differed in the speed at which tension increased during the initial period of repetitive stimulation, the rate of rise being much higher in the fast unit than in the slow one and increasing over a much wider range of stimulus frequencies (Fig. 2 A). On the other hand, with regard to maximal static tension output and the stimulus frequency at

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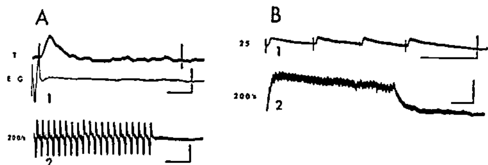


Fig 1 A Fast unit *1* Mechanical (T) and electrical (EMG) responses to a single timulus pulse Ac recording of EMG Calibrations T 15 mg EMG 100 μv time 10 ms ? EMG re ponses (dc recording) to stimulation at 200 Hz Calibrations EMG 100 μv time 20 ms
Fig 1 B Slow unit EMG (dc recording) at a rate of stimulation of 25/s (*1*) and 00/s (*2*) Calil rations EMG 100 μv time 10 ms

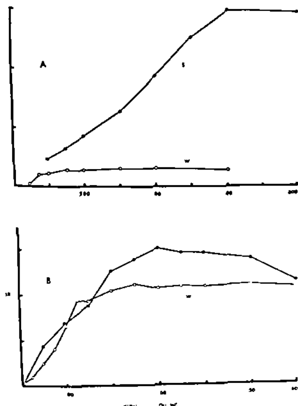


Fig 2 Rate of rise of tension (*A*) and static tension (*B*) of the fast and slow units of Fig 1 plotted against frequency of re petitive stimulation Fusion fre quency of fast unit 300/s could not be determined in slo un t

which this value was obtained the units behaved rather similarly (Fig 2 B) This means that the slov unit could operate in about the same frequency range as the fast unit

Thus with respect to their range of firing frequencies the motoneurons innervating the two types of unit would probably fall into the same group in accordance with findings by Robinson (1970) However as indicated by the values obtained for rate of tension rise in the fast and the slow unit they would contribute quite differently to the dynamic part of the various motor acts in which eye muscles are involved

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The Release of Protein in the Course of Catecholamine Secretion from Bovine Adrenals Perfused in Vitro

By

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Abstract

SERCK HANSSEN G *The release of protein in the course of catecholamine secretion from bovine adrenals perfused in vitro* Acta physiol scand 1972 *86* 289—298

The release of protein from chromaffin granules during catecholamine secretion has been studied in bovine adrenals perfused *in vitro* In response to acetylcholine an increased output of catecholamines as well as protein from the glands occurred Secretion caused a reduction in protein content of chromaffin granules corresponding to the amount of amines released The quantity of protein released during secretion from the large granule fraction the fraction containing the chromaffin granules was of a magnitude compatible with secretion of the total content of water soluble protein in the chromaffin granule These observations are consistent with an all-or none release mechanism for the adreno-medullary chromaffin granules

The concomitant release of soluble protein and catecholamines from the chromaffin granules upon stimulation of the adrenal gland is now well established and morphological and biochemical evidence for an exocytotic mechanism has been obtained (De Robertis and Vaz Ferreira 1957 Diner 1967 Banks and Helle 1965 Trifaro Poisner and Douglas 1967 Poisner Trifaro and Douglas 1967) Furthermore the catecholamines and granule protein are secreted in the same ratio as found in the soluble phase of the chromaffin granule (Schneider Smith and Winkler 1967)

It is not known whether the chromaffin granules participating in the secretory process release all or only part of their content The electronmicroscopic work by Diner (1967) on hamster medullae suggests extrusion of the total electron dense matrix of the granule at the exocytotic site In a recent study on chromaffin granules obtained from rabbit medullae before and after *in vivo* insulin induced secretio Viveros Arqueros and Kirshner (1971) also conclude that a granule relea total soluble content during secretion

Chromaffin granules obtained from *in vitro* stimulated bovine adrenals have been shown to contain less catecholamines per mg of total granule protein than granules from unstimulated glands (Serck Hanssen 1971) This observation suggested that premature granules not fully loaded with catecholamines accumulated d ring secretion but the low catecholamine protein ratio might also indicate a gradual release of the soluble phase of the granules during secretion In the present work a further investigation on the relative amounts of catecholamines and protein released upon stimulation has therefore been carried out Isolated bovine adrenals have been perfused *in vitro*, and the quantity of catecholamines and protein in cell fractions has been measured before and after stimulation with acetylcholine

Methods

The method applied for perfusion has been described in a previous paper (Serck Hanssen 1971) In the present experiment Tyrode s buffer (137 mM NaCl 2 68 mM KCl 1 80 mM CaCl 0 28 mM NaH_2PO_4 0 001 mM $MgCl_2$ 11 6 mM $NaHCO_3$ + 5 56 mM glucose) has been used as the perfusion medium The perfusion rate was adjusted to approximately 15 ml per min Stimulation was carried out as described in the previous paper the only difference being that the stimulation was halved to a period of 2 min For determination of protein and catecholamines released during secretion perfusate was collected over a period of 3 min during which stimulation occurred at the initial 2 min

Cell fractionation The weighed medullae were homogenized in 0 3 M sucrose of 0 in a volume 5 times the weight of tissue The homogenate was filtered through 2 layers of gauze to remove pieces of intact tissue The filtered homogenates were fractionated accordin to the scheme outlined in the previous paper (Serck Hanssen 1971) using a Sorvall refrigerated centrifuge and the Beckman L2 65 ultracentrifuge Chromaffin granules were isolated a noted in the text by using either the method of Smith and Winkler (1967) or the method by Banks (1965) In the latter case 1 ml of large granule suspension was layered on sucrose gradients made as follows 0 5 ml of 1 4 and 1 5 M 1 ml of 1 55 M 0 5 ml of 1 6 1 7 1 8 and 2 5 M sucrose 6 fractions were obtained from the developed gradients from the top F1 1 ml F2 1 ml (containing mainly mitochondria) F3 1 ml F4 0 5 ml F5 1 ml (containing most of the chromaffin granules) and F6 0 5 ml

Catecholamines CA were measured as the sum of adrenaline and noradrenaline by the method of Bertler Carlsson and Rosengren (1958) using a Farrand spectrofluorometer, with excitation at 390 nm and readings at 540 nm Adrenaline bitartrate (Sigma) was used as standard

Protein was determined according to Lowry *et al* (1951) Protein in 1 ml aliquots of perfusates was determined after removal of catecholamines by dialysis for 24 h against 2×500 ml of Tyrode s buffer Protein in cell fractions was precipitated with trichloroacetic acid TCA final concentration 5 % w/v The pellets were washed twice in 5 % TCA and solubilized in 1 N NaOH Washing in fat extracting solvents applied previously (Serck Hanssen 1971) has been omitted as this leads to incomplete recovery of protein in the TCA precipitates As compared with granule protein in suspensions dialysed against buffer to remove the catecholamines 5 % TCA precipitates between 75 and 90 per cent of the protein In the present experiments TCA precipitated granule protein gave a mean ratio of 2 59 µmol catecholamines per mg protein while dialysed granule protein gave a ratio of 2 72 µmol catecholamines per mg of protein

Expression of results The amount of protein in the various cell frac ions has been expressed as percentage of the protein present in the original filtered homogenates or as percentage of the protein present in the 800×*g* supernatant When the results have been corrected for lysis and efficiency of homogenization the assumption has been made that the greater part of the catecholamines are located in the chromaffin granules Thus the degree of lys s was calculated from the amount of catecholamines found in the supernatant after centrifugation at 20 000×*g* and the efficiency of homogenization was calculated from the difference in content of catecholamines in total homogenate and the 800×*g* supernatant The corrected values for the protein content in the cell fractions are based on a ratio of catecholamines to protein in chromaffin granules equal to 2 59 µmol/mg

TABLE I The distribution of protein in cell fractions of adrenal medulla expressed as percentage of total cell protein Filtered cell homogenates were centrifuged at 800 × g for 10 min in a Sorvall refrigerated centrifuge The supernatants obtained were then centrifuged at 20 000 × g for 20 min The pellets the large granule fractions were suspended in 0 3 M sucrose In 2 expts the large granule suspensions were layered over 1 6 M sucrose and chromaffin granules were obtained as pellets by centrifugation at 36 000 rpm for 60 min rotor 50 Beckman L2–65 ultracentrifuge (Smith and Winkler 1967) In 3 expts the large granule suspensions were layered on a sucrose density gradient (Banks 1965) and chromaffin granules were isolated by centrifugation at 36 000 rpm for 60 min rotor SW 65 Ti Microsomes were obtained from the 20 000 × g supernatant by centrifugation at 105 000 × g for 60 min

P=pellet SN=supernatant LG=large granules chrom gran =chromaffin granules

		of total cell protein ± S E mg cell protein per ml homogenate					
Number of exp	mg cell protein ml homogenate	800 g P	800 g SN	LG	20 000 g SN	Chrom gran	Microsomes
5	10 99 ± 0 68	18 0 ± 1 00	82 2 ± 0 86	33 2 ± 1 83	49 6 ± 2 32	10 8 ± 0 66	7 6 ± 0 51
	Corrected values	16 2 ± 1 24	84 0 ± 1 10	43 0 ± 1 41	4? 2 ± 2 40	29 4 ± 0 68	7 6 ± 0 51

A ratio of 2 59 μmol catecholamines per mg of granule protein has been used for correction for lysis and efficiency of homogenization

Results

Protein content of cell fractions from bovine adrenal medulla

The distribution of protein in various cell fractions has been estimated and the results obtained in 5 expts are given in Table I All values are based on mg protein recovered in cell fractions obtained as described under Methods and are expressed as per cent of total cell protein in filtered homogenates The greater part of the catecholamines in the cells are probably located in the chromaffin granules Thus the catecholamines found in other cell fractions arise from incomplete homogenization mechanical disruption (lysis) of granules during homogenization and incomplete sedimentation of the granules In the present experiments the efficiency of homogenization varied between 87 and 100 per cent and the degree of lysis varied between 28 and 34 per cent Taking into account the estimated amount of granule protein dislocated to other cell fractions due to lysis and incomplete homogenization the distribution of protein in the various cell fractions was changed as shown by the corrected values Table I

The bulk of *chromaffin granules sediments on centrifugation of the* 800 × g supernatant for 20 min at 20 000 × g The pellet obtained referred to as the large granule fraction LG contains in addition to the chromaffin granules lysosomes (Smith and Winkler 1966) and mitochondria and microsomes (Banks 1965) The large granule fraction represented approximately 33 per cent of total cell protein corrected value 43 per cent (Table I) The large granule fraction was further fractionated in 3 expts by the method of Banks (1965) and in 2 expts by method by Smith and Winkler (1967) The mean quantities of chromaffin in the large granules were found to be 34 0 and 30 0 per cent respectively

TABLE II The amount of protein and catecholamines recovered in the perfusates during periods of rest and stimulation Perfusates from perfused and perfused/stimulated glands were collected for a period of 3 min at the stated time intervals after beginning of perfusion *Stimulated* refers to perfusates collected when the glands were stimulated with 8 3 × 10^{-4} mmol of acetylcholine chloride *Rest* refers to perfusates collected during a period immediately before stimulation The values given are the mean values for 4 pairs of perfused glands Protein was determined in dialysed perfusates

Glands	Time of collection of perfusate	µmol CA per min	mg protein per min	µmol CA per mg prot	µmol CA per mg prot
Perfused	45 min	0 18	1 20	0 15	
	100	0 19	0 63	0 23	
	160	0 11	0 40	0 27	
Stimulated					
Rest	45	0 19	1 20	0 18	
	100	0 19	0 60	0 32	
	160	0 14	0 41	0 33	
Stimulated	45	1 21	1 12	1 30	6 69
	100	0 86	0 69	1 18	4 05
	160	0 70	0 51	1 01	5 03

The ratio of catecholamines (CA) to protein during stimulation based on values of catecholamines and protein corrected for the amount of the compounds released during rest

2 methods indicating a good correlation between the methods The values for the content of chromaffin granules given in Table I and III are the means of the 5 expts Thus chromaffin granules constituted about 11 per cent of total cell protein (Table I) and about 33 per cent of the large granule fraction (Table III) When considering the catecholamines and thus the granule protein of the large granule fractions not sedimenting with the chromaffin granules the chromaffin granules were found to represent as much as 29 per cent of total cell protein and 68 per cent of the large granule fraction

Protein recovered in perfusates of stimulated and control glands

Isolated bovine adrenals were perfused *in vitro* with Tyrode s buffer in a retrograde fashion as already described (Serck Hanssen 1971) Both glands from the same animal were perfused one serving as control while the other was stimulated with acetylcholine (1 mmol of acetylcholine chloride in total) As shown by Schneider Smith and Winkler (1967) considerable amounts of protein and catecholamines were released during the first 15 min of perfusion This fell to a nearly constant value within 45 to 60 min of perfusion In the present experiments the release of protein and catecholamines during rest varied between 0 3—1 35 mg/min and 0 04—0 25 µmol/min respectively depending on the size of the glands

Acetylcholine stimulated the secretion of catecholamines and protein As shown in Table II the ratios of catecholamines to protein released during stimulation were higher than the ratios during rest Corrected for the amount of amines and protein releasing during resting periods even higher ratios were obtained The corrected

ratios agree well with the ratios obtained for perfusates from stimulated adrenals by Banks and Helle (1965) and Schneider Smith and Winkler (1967) and which have been shown by the latter authors to be similar to the ratio of catecholamines to protein found for the soluble lysate of chromaffin granules Thus under the present experimental conditions protein and catecholamines were released from the glands upon stimulation in a ratio corresponding to that in which they are found in the soluble phase of the chromaffin granules The low catecholamine protein ratios in the perfusates during rest indicated however that throughout perfusion also extragranular proteins were released from the glands These proteins might be of cortical as well as medullary origin

Protein content of cell fractions obtained from stimulated and control perfused glands

Cell fractions isolated as described under Methods were obtained from the perfu ed and perfused/stimulated glands described in the previous section The relative distribution of protein in cell fractions from the perfused control glands agreed well with the values obtained for non perfused glands (Table I) with the exception of the microsome fraction (Table III) The microsome fractions from perfused glands made up significantly more of total cell protein than the microsome fractions from non perfused glands As the ratios of catecholamines to protein for the microsomes from non perfused glands and perfused glands were 0 17 $\pm$ 0 020 (n = 5) and 0 18 $\pm$ 0 017 (n = 4) μmol/mg respectively the increase in microsomal protein in perfused glands was probably not due to the presence of more chromaffin granules The increase in microsomal protein was also evident when microsomal protein was expressed as per cent of protein in the 20 000 $\times g$ supernatant and a more likely explanation seems therefore to be that loss of cytoplasmic proteins took place during perfusion

Comparing the relative distribution of protein in cell fractions from perfused glands and perfused glands stimulated with acetylcholine differences were observed for the large granule fractions the fractions of chromaffin granules and the microsome fractions (Table III) As small but insignificant decline in protein content of the large granule fractions was found in the stimulated glands Comparing the corrected values for protein content in the large granule fractions from perfused and perfused/stimulated glands the difference was significant The sum of protein recovered in the 800 $\times g$ pellet the large granule fraction and the 20 000 $\times g$ supernatant exceeded total cell protein for some of the perfused and perfused/stimulated glands while the sum of protein in the large granule fractions and the 20 000 $\times g$ supernatant always were in good agreement with the amount of protein found in the 800 $\times g$ supernatant Protein in the large granule fractions has therefore also been expressed as per cent of protein in the 800 $\times g$ supernatant (Table III) Comparing these values when corrected the large granule fractions from stimulated glands contained about 14 per cent less protein than the corresponding fractions from the controls

TABLE III Protein content in cell fractions from non perfused, perfused and perfused stimulated (F5) were isolated on a sucrose density gradient as described by Banks (1965). The (SN) per cent of the large granule fraction (LG) or as per cent of the 20 000×g

Glands	Number of exp	mg tcp/ ml homogenate	LG % of tcp	% of 800 g SN
Non perfused	5	10.99±0.68	33.2±1.83	40.6±1.96
Perfused	4	8.98±0.36	*35.0±0.82	41.8±1.44
Stimulated	4	7.57±0.54	*32.8±1.11	38.5±1.04
Corrected values*				
Non perfused			43.0±1.41	51.0±1.41
Perfused			*44.3±1.31	*51.8±1.31
Stimulated			*39.0±0.58	*44.5±1.19

* A ratio of 2.59 μmol catecholamines per mg of granule protein has been used for correction of lysis and efficiency of homogenization

The quantity of chromaffin granule protein obtained in F5 on the sucrose density gradients (see Methods) was about 25 to 28 per cent less in stimulated glands than in the controls as seen from the granule protein expressed as per cent of total cell protein (Table III). The mean catecholamine—protein ratios for the chromaffin granules of the perfused and perfused/stimulated glands were 2.34 and 1.93 μmol CA/mg protein respectively.

As compared to the control perfused glands the microsome fractions from the stimulated glands represented significantly more of total cell protein and more of the protein in the 20 000×g supernatant (Table III). The ratio of catecholamines to microsomal protein was found to be 0.13±0.006 (n=4) μmol/mg. Although not significantly lower than the ratio for the microsomes from perfused glands (see above), the ratio of 0.13 μmol catecholamines per mg of protein might indicate an accumulation during secretion of protein sedimenting at 105 000×g.

Acetylcholine induced secretion of protein from perfused adrenals

In the present experiments a spontaneous release of catecholamines as well as protein occurred during perfusion (Table II). Equal amounts of protein were released per min from the control glands and the stimulated glands during resting periods (Table II). Any difference in the quantity of protein in the various cell fractions from the stimulated and control glands may thus be attributed to granule protein discharged during acetylcholine induced secretion. An approximate estimate of the quantity of protein released from the large granule fraction during induced secretion can be made assuming discharge of the total granule content of water soluble protein and amines but no loss of protein from the other organelles, i.e. mitochondria and lysosomes. Thus released granule protein equals

Lg protein × % granule protein × % amines release × % granule soluble protein.

According to Hillarp (1958) 78 per cent of the granule protein is water-soluble while Helle (1971) has reported a value of 66 per cent soluble granule protein

bovine adrenal medullae Cell fractions were obtained as described in Table I Chromafin granules results are expressed as per cent of total cell protein (tcp) per cent of the 800 × g supernatant supernatant Values are means ± S E

Chromaffin granules		Microsomes	
of tcp	% of LG	% of tcp	% of 20 000 g SN
10 8 ± 0 66	32 8 ± 1 60	[1]7 6 ± 0 51	15 4 ± 0 60
10 0 ± 0 91	28 3 ± 2 02	[1]10 3 ± 0 25	21 0 ± 0 91
7 5 ± 1 04	21 3 ± 2 17	13 5 ± 0 50	25.5 ± 0 96
29 4 ± 0 68	68 4 ± 2 50		18 0 ± 0 71
30 5 ± 1 37	68 3 ± 1 49		24 5 ± 1 19
21 8 ± 0 75	56 5 ± 1 19		27 8 ± 1 03

[1] $p < 0\ 005$ [2] $p < 0\ 1$ [3] $p < 0\ 05$ [4] $p < 0\ 001$

The content of catecholamines in the perfused and perfused/stimulated glands was 0 93 ± 0 08 and 0 64 ± 0 07 μmol CA/mg of total cell protein respectively Thus 32 per cent of the amines was released in response to acetylcholine Comparing the amount of protein in the large granule fractions for each pair of the perfused and perfused/stimulated glands, the secretion caused a decline in the large granule protein amounting to 6 5 and 7 3 per cent using the values for the large granule protein expressed as per cent of total cell protein and 800 × g supernatant respectively (Table IV) Although relatively small this acetylcholine induced reduction in protein nevertheless corresponded well with the theoretical estimates of secreted protein calculated according to the above relation The difference in large granule protein from control and stimulated glands when corrected for lysis and incomplete homogenization was about 12—14 per cent which also was in agreement with the theoretical values (Table IV)

TABLE IV Acetylcholine induced discharge of protein from the large granule fraction of adrenal medullae perfused *in vitro* Theoretical estimates of granule soluble protein discharged from the LG during secretion has been calculated on basis of secretion of 32 per cent of the catecholamines in the perfused glands containing granule protein amounting to 78 per cent of the LG protein (Table III) Calculations have been made for a content of granule water soluble protein equal to 78 per cent (Hillarp 1958) and 66 per cent (Helle 1971) of total granule protein Values are means ± S E

	% difference in protein content in LG from perfused and perfused/stimulated glands		Estimates of granule protein discharged from the LG	
			78 soluble gran prot	66 soluble gran prot
Measured values	6 5 ± 2 06	7 3 ± 2 39[b]	7	6
Corrected values	11 8 ± 2 33	14 0 ± 0 58[b]	17	14

[a]Large granule protein expressed as per cent of total cell protein (Table III)
[b]Large granule protein expressed as per cent of the 800 × g supernatant protein (Table III)

In good correlation with output of about 1/3 of the amines in response to acetylcholine the stimulated glands contained 25—28 per cent less granule protein in chromaffin granules than the control glands (Table III) As the emptied granule vesicles sediment with the large granule fraction (Poisner et al 1967) the percentage of chromaffin granules remaining in the large granules of the stimulated glands can also be estimated from the following relation

$$\frac{\text{granule protein—protein of emptied granules}}{\text{LG protein—soluble granule protein released}} \times 100$$

The granule protein represented 28 per cent of the large granule protein in the perfused glands (Table III) After release of 32 per cent of the amines with a concomitant release of the water soluble protein (78 per cent of total granule protein) the granule protein would according to the above relation represent 20 per cent of the large granule protein The granule protein was actually found to account for 21 per cent of large granule protein in the stimulated glands (Table III)

Thus secretion caused a reduction in the content of protein in the large granule fraction as well as in the content of chromaffin granules corresponding to a discharge of the total water soluble content of the chromaffin granule

Discussion

The present experiments confirm earlier observations in that stimulation of the isolated perfused bovine adrenal induce release of protein and catecholamines in a ratio similar to that found for the soluble phase of the chromaffin granule (Banks and Helle 1965 Schneider Smith and Winkler 1967) The acetylcholine induced secretion caused an average 32 per cent reduction in the tissue content of catechol amines when related to total cell protein In accordance with a parallel release of catecholamines and protein from the granule the secretion also produced a decline in the protein content of the large granule fractions of the stimulated glands Furthermore the amount of protein released indicated that negligible loss of protein from the large granule fractions besides that of the water soluble protein of the granules occurred Thus emptied and/or partially emptied granules sedimented with the large granule fraction The amount of secreted catecholamines could be accounted for by a corresponding reduction in the content of chromaffin granules in the large granule fraction Although the granules retained in the cell after secretion contained less catecholamines per mg of total granule protein an accumulation of partially emptied granules during secretion thus seemed unlikely Therefore the low catecholamine protein ratio for the remaining granules was probably due to enrichment in premature granules rather than contamination by partially emptied granules in agreement with observed changes in their biochemical and morphological characteristics (Serck Hanssen and Helle 1972) The present observations thus provide additional evidence for an all or none release mechanism for the chromaffin granules of the adrenal medulla as suggested by Viveros et al (1971)

The mechanism of secretion involves fusion of the granule membrane and the cell membrane with extrusion of the granule content at the cell surface (Banks and Helle 1965 Diner 1967, Poisner *et al* 1967) The emptied granule vesicles are recaptured by means of vesiculation giving rise to microvesicles of various sizes (Douglas and Nagasawa 1971) The retained vesicles sediment with the large granule fraction at centrifugal forces of 20 000×*g* (Poisner *et al* 1967 Viveros *et al* 1969 Serck Hanssen 1971) Due to the different sizes of the vesicles (Douglas and Nagasawa 1971) one might expect some of the smaller vesicles to sediment only at higher centrifugal forces In fact secretion produced a small but significant increase in protein sedimenting at 105,000×*g* As secretion also causes an increase in the 105 000×*g* pellet of the granule bound enzyme dopamine *β* hydroxylase (Viveros *et al* 1969) a small fraction of the emptied vesicles seems to be present in the microsome fraction

In preliminary experiments it was observed that 5% TCA gave incomplete precipitation of total granule protein This incomplete precipitation is probably due to the high content of polar amino acids in chromogranin A (Helle and Serck Hanssen 1969) Acidic proteins obtained from monkey brain with a rather similar content of polar amino acids are soluble in trichloroacetic and perchloric acid (Reichelt 1971) The obligatory presence of methanol in the acetic acid during fixation of chromogranin A on polyacrylamide gels has also been noticed (Helle 1971) It is unlikely however, that the observed differences in protein content in cell fractions from perfused and perfused/stimulated glands are due to incomplete TCA precipitation of granule protein Thus the incomplete precipitation of granule protein would influence the protein recovery in all fractions similarly and a recovery of protein equal to or very close to 100 per cent was found throughout the whole subcellular fractionation procedure in all the experiments Furthermore one might expect incomplete precipitation to produce a greater error in cell fractions of the perfused glands containing more chromaffin granules than the corresponding fractions of the stimulated glands Incomplete precipitation of granule protein would accordingly rather diminish than enhance quantitative differences in protein content in the cell fractions from the perfused and perfused/stimulated glands

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Cardiac Noradrenaline Turnover and Urinary Catecholamine Excretion in Trained and Untrained Rats during Rest and Exercise

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Abstract

ÖSTMAN, I., N. O. SJÖSTRAND and G. SWEDIN. *Cardiac noradrenaline turnover and urinary catecholamine excretion in trained and untrained rats during rest and exercise.* Acta physiol. scand. 1972. 86. 299—308.

Rats were trained by daily swimming (1—2 h) during 15 weeks. The training caused a significant increase in heart weight. Cardiac noradrenaline (NA) and adrenal catecholamine (CA) contents were significantly increased in trained animals. When exposed to acute prolonged exercise the endogenous levels of cardiac NA decreased in untrained but not in trained rats. Resting cardiac NA turnover, as measured by rate of disappearance of exogenous ^{3}H-NA, was slower in trained than in untrained rats ($T_{1/2}$ = 52 h and 20 h, respectively). The NA turnover increased during exercise but still remained slower in trained rats. Figures were obtained indicating a significantly lower initial uptake of exogenous NA in the hearts of trained rats. Resting excretion of CA in the urine was not different in trained and untrained rats while the increase seen after a period of exercise was much greater in the untrained animals. The results indicate that chronic physical training induces a functional adaptation of the sympathicoadrenal system leading to a better transmitter economy during exercise. The nature of this adaptation remains, however, unsettled.

Little is known about the adaptation of the sympathico-adrenal system to chronic physical exercise. One factor that has been studied in order to reveal changes in this system is the content of adrenergic transmitter in different organs and of CA in the adrenal glands (*cf.* Östman and Sjöstrand 1971 b). In previous studies on the rat and guinea pig (Östman and Sjöstrand 1971 a, b) only minor changes in cardiac NA content were found in the trained animals. However, small changes in the tissue content of NA might reflect considerable changes in the dynamics of cardiac NA. Accordingly, the turnover of radioactively labeled NA in the hearts of trained and untrained rats during rest and exercise has been investigated in the present study.

Since it is known that acute physical exercise increases the urinary excretion of CA (Euler and Hellner 1952), it was also of interest to compare the urinary excretion of CA by the trained animals with that by the untrained animals after a period of exercise. In addition the effect of prolonged training on the CA content of the adrenals was measured.

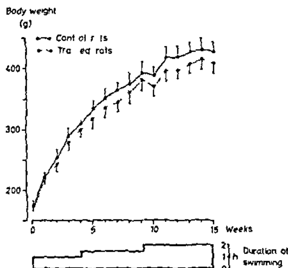


Fig 1 Increase of body weight of trained and untrained (control) rats during the training period mean ± S D The duration of the daily swimming period is indicated in the figure Symbols see figure

Material and Methods

68 mal Sprague Dawley rats initially weighing about 175 g were used The animals were kept at a room temperature of 24 C and received food and water ad lib 34 of these rats were submitted to daily exercise during 15 weeks the rest served as control The animals were exercised by swimming in baths 70 × 44 × 53 cm with a depth of water of about 42 cm. 8—10 rats were swimming simultaneously in the same bath The water was kept at a temperature of 35 ± 1 C The period of swimming was initially 1 h and was gradually increased to 2 h/day as illustrated in Fig 1

Unless otherwise stated all figures referring to organ weights and endogenous levels of CA are based on the values of the resting groups For the final experiments the groups of exercised rats and the control groups were each randomly divided into 2 subgroups of 16 animals each One of these groups was used for *determination of NA turnover* during resting conditions and the other during exercise The animals in the resting groups were killed 20—30 h after the last exercise period at 4 8 12 or 16 h after an injection of 10 μCi l ^{3}H NA (Amersham Int sp act 4 1 Ci/mmol) in 0 5 ml saline in the tail vein

The determination of ^{3}H NA turnover during exercise was carried out with an intermittent exercise program which was designed (*cf* Fig 3) so as to permit the longest po sible observation time without exceeding the limited capacity of the untrained rats to withstand prolonged swimming The swimming was started within 5 min after the injecton of l ^{3}H NA and the animals were killed after 4 8 12 and 16 h of exercise

Collection of urine The determinations of the *urinary excretion* of CA wer made during the 14th week of the training period 12 exercised rats and 12 controls were u ed The urine was collected in metabolic cages where the animals were kept individually over a period of 2? h The first sampling period was preceded by 24 h of rest the second was started immediat ly after 2 h of swimming The rats were carefully blotted after the swimming periods The sampling was made simultaneously on exercised rats and controls to exclude the possible influence of external factors Room temperature was kept at 22—24 C

CA extraction and determination The animals were sacrificed by bleeding under light ether anesthesia The hearts and adrenal were taken out cleaned and weighed The *hearts* were homogenized with an Ultra Turrax apparatus in ice cold 0 4 N perchloric acid NA was extracted as previously described (Swedin 1971) and the endogenous amounts determined fluorimetrically according to Chang (1964) The *adrenal glands* were ground with chemically pure quartz sand in 2 ml of 0 4 N perchloric acid The extracts were oxidized and the NA and A was determined fluorimetricall according to Euler and Lishajko (1961) Total radioactivity expressed as cpm was determined in aliquots from the hearts and plasma, as well as from the alum na eluates of the heart samples after dilution in 10 ml Inst gel (Packard)

TABLE I Initial and final body weight (b. w.) final heart weight heart ratio and adrenal weight (paired organs) of trained and untrained (control) rats during resting conditions Mean ± S.E. o $p > 0.2$ ** $p < 0.01$ ** $p < 0.001$ n = number of animals

	n	Untrained	Trained	of control
Initial b. w. (g)	32	173 ± 2	175 ± 1	101
Final b. w. (g)	32	427 ± 4	407 ± 4 * *	95
Heart weight (g)	16	1.28 ± 0.0[illegible]	1.37 ± 0.0[illegible] **	107
Heart ratio (g/100 g b. w.)	16	0.30 ± 0.003	0.33 ± .004	110
Adrenal weight (mg)	16	41 ± 1	45 ± 1 *	110

using an Intertechnique Abac SL 40 Liquid Scintillation Spectrometer Quenching was monitored by internal standards Specific activity of the heart NA (cpm/μg NA) was calculated from the radioactivity in the alumina eluates without correction for metabolites

Urinary CA were extracted and fluorimetrically determined according to Euler and Lishajko (1961) The CA values are expressed as μg free base

Statistical analysis was performed using the method of least squares and Student's t test The turnover curves were compared by means of calculating a t value based on all consecutive means of the 2 groups [1]

Results

General observations The behaviour of the *trained rats* was little affected by the daily period of swimming as well as by the final experimental periods of exercise It was noted that the animals almost immediately started eating and moving after they had returned to their cages The *untrained rats* appeared to be unaffected by the 2 h period of swimming preceding the determination of urinary CA (see methods) but they were severely exhausted after the 8 h of continuous swimming in the final experimental period in fact 3 untrained rats died from exhaustion after 7—8 h of swimming

Animal and organ weights The increase of b. w. of the trained rats during the experimental period was smaller than that of the controls (Fig 1) Initial and final b. w. absolute heart weights heart weights related to b. w. as heart ratio (g/100 g of b. w.) and weights of adrenal glands are given in Table I The final b. w. of the trained rats was significantly lower than that of the controls ($p < 0.01$) The trained rats had greater heart weights ($p < 0.01$) heart ratio ($p < 0.001$) as well as adrenal weights ($p < 0.01$) when compared with the controls

[1]

$$t = \frac{(\bar{Y}_{4c} - \bar{Y}_{4e}) + (\bar{Y}_{8c} - \bar{Y}_{8e}) + (\bar{Y}_{12c} - \bar{Y}_{12e}) + (\bar{Y}_{16c} - \bar{Y}_{16e})}{\sqrt{S_p^2 \frac{a}{n}}}$$

$\bar{Y}_{jk}$ is the mean of the observations made at the time j (j = 4 8 12 16 h) on animals from the group k (k = c e c = controls e = trained animals) S_p = pooled variance n = number of obs on which mean is based a = number of $\bar{Y}_{jk}$ Degrees of freedom = a(n 1)

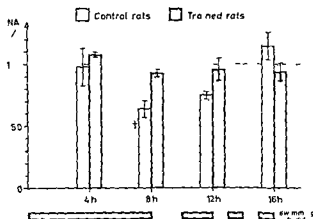


Fig 2 Effect of acute prolonged swimming on total cardiac NA content in trained und untrained (control) rats The columns indicate total amount of NA in % (±S E) of the mean of resting animals controls or trained respectively at the same point of time during the final experiment The swimming schedule during the final experimental period is indicated in the figure + indicates the value for one of the rats that died of exhaustion during the experiment Differences from resting value * $p < 0.05$ * $p < 0.01$

CA contents

Heart A definite increase in cardiac NA content was observed in the trained rats evident as a 25 % increase of the total amount of NA ($p < 0.001$) as well as a 16 % increase in the organ concentration of the amine ($p < 0.01$) (Table II)

When the NA content of the hearts was determined after the period of swimming that comprised the final experimental period a striking difference was found between the trained and untrained animals This is illustrated in Table III and Fig 2 After 4 h of swimming the NA content of the heart was unchanged in both groups However after 8 h there was a significant decrease (36 %) in the NA content of the hearts from the untrained animals ($p < 0.01$) whereas the NA content of the hearts from trained animals did not decrease After allowing the animals periods of intermittent rest the cardiac NA content gradually recovered to normal levels in the untrained rats

Adrenals The adrenal glands of trained animals contained 20 % more A ($p < 0.05$) and 94 % more NA ($p < 0.001$) than adrenals from control animals In addition NA comprised a greater proportion of the total CA in the adrenals of trained animals ($p < 0.001$) (Table II)

Table II CA content of heart and adrenals of trained and untrained (control) rats during resting conditions Mean ± S E n = 16 when not other wise indicated * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

	Heart Total NA µg	NA µg/g	Adrenals A /g	NA /g	% NA of total CA
Controls	0 93 ± 0 03	0 73 ± 0 03	35 6 ± 1 7	6 25 ± 0 58	15 ± 1
Trained	1 16 ± 0 04***	0 85 ± 0 03**	42 8 ± 2 4* (n = 5)	12 14 ± 1 03*** (n = 5)	22 ± 1**
% of control	125	116	120	194	147

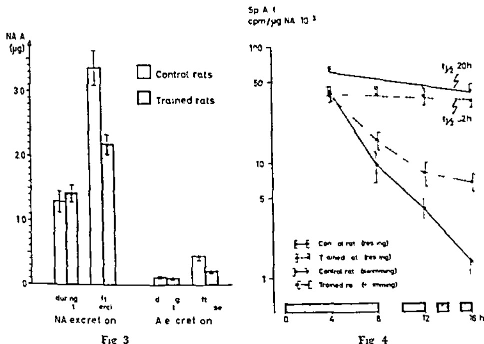


Fig 3 Fig 4

Fig 3 Urinary CA excretion of trained and untrained (control) rats during rest and after 2 h of exercise The values are given as µg CA/kg b w and 22 h mean ± S E Symbol see figure Differences from controls ** $p < 0.001$ differences from resting value × $p < 0.05$ × × $p < 0.01$ × × × $p < 0.001$

Fig 4 Disappearance rate of specific activity of NA in the hearts of trained and untrained (control) rats during rest and exercise after i v injection of 10 µCi ^{3}H NA in 0.5 ml saline at 0 h Mean ± S E Straight lines are drawn according to the method of least squares Symbols see figure The swimming schedule is indicated in the figure

Urinary CA excretion During resting conditions there was no difference in CA excretion between trained and untrained animals After a 2 h period of swimming the excretion of CA increased significantly in both trained and untrained rats (Fig 3) This increased CA excretion was however significantly lower in the trained rats ($p < 0.01$) compared with the controls (Table IV Fig 3)

TABLE III Total cardiac NA (µg) of trained and untrained (control) rats during rest and acute longlasting exercise (swimming program indicated in Fig 2) Mean ± S E n = number of expts Differences from resting controls $p < 0.05$ * $p < 0.01$

	4 h	8 h	12 h	16 h
Controls resting	0.97 ± 0.09 (n = 4)	0.98 ± 0.06 (n = 4)	0.91 ± 0.06 (n = 4)	0.86 ± 0.04 (n = 4)
during exercise	1.02 ± 0.18 (n = 4)	0.62 ± 0.06 (n = 3)	0.68 ± 0.03* (n = 3)	0.99 ± 0.09 (n = 3)
Trained resting	1.13 ± 0.05 (n = 4)	1.14 ± 0.06 (n = 4)	1.26 ± 0.08 (n = 4)	1.16 ± 0.04 (n = 4)
during exercise	1.25 ± 0.03 (n = 4)	1.05 ± 0.04 (n = 3)	1.21 ± 0.11 (n = 4)	1.01 ± 0.07 (n = 4)

TABLE IV Urinary CA excretion of trained and untrained (control) rats during rest and after exercise (2 h of swimming) Mean ± S.E. n = 12 ° p > 0.2 ** p < 0.01 (differences from controls)

	Controls	Trained	% of control
During rest			
A µg/22 h	0.05 ± 0.004	0.05 ± 0.01	100 %
µg/22 h kg b. w.	0.12 ± 0.01	0.11 ± 0.02	92 %
NA µg/22 h	0.62 ± 0.06	0.60 ± 0.05	97
µg/22 h kg b. w.	1.29 ± 0.18	1.42 ± 0.13	110
After exercise			
A µg/22 h	0.20 ± 0.03	0.10 ± 0.07**	50
% of resting value	400	200 %	
µg/22 h kg b. w.	0.45 ± 0.06	0.22 ± 0.04**	49 %
of resting value	385 %	200 %	
NA µg/22 h	1.39 ± 0.12	0.91 ± 0.08**	65 %
of resting value	224 %	152	
µg/22 h kg b. w.	3.17 ± 0.27	2.18 ± 0.15**	69 %
of resting value	246 %	153 %	

NA turnover

Heart The organ content of radioactive NA (expressed as specific activity) during rest as shown by the 4 h value was significantly lower in the trained rats (62 % of the controls $p < 0.01$) The curves of the radioactivity (Fig 4) as a function of time were significantly different ($p < 0.01$) mainly because of the higher level of radioactivity in the controls The intercept ($x = 0$) but not the slope was proven to be significantly different ($p < 0.05$) The half time period ($T_{1/2}$) for the disappearance of radioactive NA was 20 h in the controls and 52 h in the exercised rats Exercise increased the rate of turnover of cardiac NA in both groups of rats During exercise the rate of turnover of NA was significantly higher in the untrained rats ($p < 0.01$) Between 4 h and 8 h of continuous swimming the $T_{1/2}$ was about 2 h for the controls and 3 h for the trained animals After shifting to intermittent exercise the rate of NA turnover decreased but still remained higher in the untrained animals (Fig 4) It should be mentioned that the rate of disappearance of cardiac radioactivity in untrained animals is higher expressed as total radioactivity than as specific activity due to the simultaneous decrease in endogenous NA The ratio between the radioactivity in the homogenates and the alumina eluates was not markedly different in trained and untrained animals indicating that the proportion of radioactive methylated metabolites was not altered

Discussion

In previous investigations (Östman and Sjöstrand 1971 a b) it was found that the endogenous levels of NA in the hearts of trained animals tended to be higher than the levels in hearts from control animals In the present study a more definite in

crease in cardiac NA is observed after training. Furthermore, the previous observation (Östman and Sjöstrand 1971 b) that the adrenal A increases in exercised rats is confirmed. In the present report, however, we find a significant increase in adrenal NA as well.

When the animals were exposed to acute longlasting exercise further differences between trained and untrained animals have been revealed. Thus after 8 h of continuous swimming the cardiac NA of untrained but not of trained animals is significantly decreased. Furthermore the increased turnover of CA during exercise as measured both by the rate of disappearance of cardiac ^{3}H NA and by increased urinary excretion of CA is significantly lower in trained animals. It has also been found that the uptake of exogenous NA during resting conditions seems to be lower in trained rats. The rate of cardiac NA turnover of trained animal at rest is less than half of that of untrained rats.

The reason for the increase in cardiac NA caused by training can at present only be a matter of speculation. One possibility could be an increased number of nerve terminal and/or a larger NA content in each terminal. One factor which might influence the amount of NA in each terminal is the actual nerve impulse flow. Thus Bhagat (1967) reported a significantly increased cardiac NA level in rats after a treatment with ganglionic blocking agents. Indications of a decreased sympathetic tone in trained animals might be the bradycardia (Tipton 1965) as well as the lower rate of cardiac NA turnover observed in the present study. However, the increased levels of NA persist even during acute prolonged exercise which causes a circa 17 fold increase in rate of NA turnover. This indicates that other factors, *e g* effectiveness of synthesis, storage and re uptake of NA might also be of importance in maintaining the increased levels of NA in the hearts of trained rats. It is interesting to note that the decrease in cardiac NA of untrained rats during the prolonged acute exercise is observed at the same time as signs of severe exhaustion occur.

In the previous study (Östman and Sjöstrand 1971 b) an increase in both adrenal weight and adrenal A was observed in the trained animals. It is well known that the weight of the adrenal glands increases after various types of stress (*cf* Selye 1953); this increase is in rodents apparently mainly due to an increase in the width of the fasciculata zone (*cf* Christian 1959). In our earlier report we tentatively suggested that the increase in adrenal A could be due to an influence of corticosteroids from a hyperactive cortex (*cf* Wurtman 1966). In the present report the increase in adrenal CA is more marked for NA than A. This makes the above mentioned hypothesis less plausible and points at other explanations, *e g* a nerve impulse induced increase of CA synthesis (*cf* Östman and Sjöstrand 1971 b). An increase in CA content in the adrenal medulla could be due either to an increased content of CA per granule, an increased number of granules per cell or an increased number of chromaffin cells. Mitoses of chromaffin cells have in fact been demonstrated even in the adult rat although they are very infrequent (Malvaldi, Mencacci and Viola Magni 1968). It is however at present impossible to rule out any of the above mentioned possibilities.

The design of the urinary sampling experiment was chosen as to minimize the possible effects of cold stress on the rats It should be mentioned that the method of urinary sampling used does not allow correction for losses of urine and CA excreted in the bath during the exercise period After acute exercise the increase in urinary excretion of both A and NA is significantly smaller in the trained animals This indicates that the effect of physical training involves both parts of the sympathico adrenal system Since urinary excretion of NA mainly reflects NA released from adrenergic nerves (*cf* Euler 1956) the finding that the amount of NA excreted by trained animals after exercise is lower than that excreted by controls suggests that in trained animals a lower sympathetic activity is required in order to maintain for example vascular and metabolic functions Alternative explanations of the differences between the amounts of NA excreted by the two groups of animals after exercise cannot however be excluded For example training might cause changes in the metabolism of the CA, secondly since the control rats were unaccustomed to swimming they may have excreted more NA due to the initial unspecific stress of being forced to swim

The rate of disappearance of exogenously administered tritiated NA from adrenergically innervated organs has been widely used as a means for estimation of NA turnover (Montanari *et al* 1963 and others) Although this method probably gives only an approximate value of the absolute turnover any change in the rate of NA turnover would be reflected in an altered decay of labelled NA

After administration of exogenous NA the initial NA uptake in organs is known to be influenced by factors as the plasma concentration of NA, the perfusion of the organ and the density of the innervation (Kopin Gordon and Horst 1965), as well as impulse flow in the adrenergic nerves (Chang and Chiueh 1968) In the present study there was no significant difference in the plasma concentrations of total radioactivity between the different groups Since the amount of radioactivity at a certain time is a function of both uptake and release and since the rate of loss of ^{3}H NA from the heart is lower in trained animals it seems highly probable that the significant difference in amount of cardiac radioactivity at 4 h is a result of a lower initial uptake in the trained animals The slopes of the initial parts of the turnover curves during exercise also seem to indicate a higher uptake in the hearts of the untrained rats Possible differences in heart perfusion due to *e g* a lower heart rate in the trained rats are not likely to be great enough to explain the initial difference in total ^{3}H NA uptake especially not when the increase in myocardial tissue of the trained rats is considered (*cf* Sonnenblick 1970) Neither is it probable that the density of innervation is higher in the controls On the contrary it is in the trained animals we observe an increase of cardiac NA concentration even if this does not necessarily mean a denser adrenergic innervation

Contradictory results concerning the effect of impulse flow on the neuronal uptake of NA have been reported (Chang and Chiueh 1968 Haggendal and Malmfors 1969) Since it is known that the turnover of NA is dependent on the nerve impulse flow (*cf* Bhagat 1967) the higher rate of NA turnover observed in the untrained

rats at rest indicates a higher activity in the cardio-accelerator nerves (see below) which according to Chang and Chiueh (1968) is compatible with the greater uptake of NA in the hearts of these animals

The turnover rate of cardiac NA found in the untrained animals at rest is in accordance with that previously reported for rats of the same size sex and strain (Swedin 1970) Chronic physical training induced a marked decrease in rate of NA turnover during rest as indicated by the more than two fold increased $T_{\frac{1}{2}}$- value This value (52 h) seems to be far higher than any previously reported for the rat (cf Geffen and Livett 1971) The most likely explanation for this slow cardiac NA turnover appears to be a lowered sympathetic tone to the heart During continuous exercise the cardiac NA turnover is considerably increased in both trained and untrained rats but the trained rats still maintain a lower turnover than the controls This seems to indicate that the trained animals also during exercise require a lower degree of sympathetic stimulation of the heart

Taken together the present results indicate that prolonged physical training in rats induces a functional adaptation of the sympathicoadrenal system which is reflected by reduced demands for CA release from the adrenal medulla and sympathetic nerve terminals The present finding of a lower cardiac NA turnover is not surprising in view of the bradycardia and the probable increase in stroke volume of the athletic heart

The overflow of NA from the heart probably constitutes a minor fraction of the total amount of NA excreted in the urine This assumption is based on the fact that there is no difference in resting NA excretion between untrained and trained rats although the latter have a lower rate of cardiac NA turnover Therefore the great difference between the groups in urinary NA excretion after exercise seems to indicate that the adaptation to the increased demands during exercise may also involve other sympathetic nerve terminals in addition to those in the heart as for example those in the peripheral vascular beds Physical training may perhaps also decrease the metabolic requirements for CA during acute exercise in a similar way as cold adaptation reduces the requirements during cold stress (cf Hsieh and Carl on 1957 Leduc 1961)

The finding that the urinary CA excretion in trained animals is not altered at rest could be explained by the adaptive mechanism not operating during rest except in the heart or by an adaptation e g in the amount of transmitter released being masked by other factors resulting in an unaltered total overflow of transmitter

Whether the adaptation is primarily due to a change in the central regulation of the sympathico adrenal system or to a peripheral change in the neuroeffector system e g an increased sensitivity or response of the effector cells or altered mechanisms for release and uptake of neuro transmitter can at present only be a matter of speculation

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Muscle Metabolites with Exhaustive Static Exercise of Different Duration

By

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Abstract

KARLSSON, J. and B. OLLANDER. *Muscle metabolites with exhaustive static exercise of different duration.* Acta physiol. scand. 1972. *86.* 309—314.

Concentrations of ATP, CP, glycogen and lactate were determined in the lateral portions of the thigh at rest and at different percents (75, 50, 25 and 10 %) of the individual maximal voluntary isometric contraction (MVC). Endurance times were 0.5, 1.6, 5.8 and 38.7 min respectively. A phosphagen depletion of 15 mmol × kg^{-1} wet muscle and a lactate accumulation of 20 mmol × kg^{-1} was obtained only with the 50 % MVC which is similar to what is observed with short time exhaustive bicycle exercise. At both higher and lower intensities phosphagen depletion and lactate accumulation were less.

Dynamic muscle exercise in man, such as bicycling and running, has been studied extensively with regard to both circulatory and metabolic demands. Static exercise, however, has not been investigated to the same extent. Although various circulatory responses to static muscle contractions have been examined (Humphreys and Lind 1963, Lind *et al.* 1964, 1966, 1967 and Freyschuss 1970) few metabolic studies appear in the literature (Bergström *et al.* 1970). This is the case in spite of such intriguing characteristics as a high energy turnover without performing any external mechanical work. Moreover, there is, except for very mild tensions, an inadequate oxygen supply due to the occlusive effect on the capillary bed caused by sustained muscle contraction.

The present study was undertaken to evaluate the degree of lactate accumulation and phosphagen depletion in the active muscle groups of man at different percentages of the maximal static force sustained to exhaustion.

Subjects, Procedure and Methods

3 healthy male physical education students participated in this study. Some of their characteristics are presented in Table I. Static exercise was performed in a chair constructed for leg extension exercise. The angle between femur and tibia was 90°. The force generated was measured via a strain gauge located on an immovable iron bar under the subjects' arch.

TABLE I Pertin nt individual anthropological and physiological data

Subject	Age years	Weight kg	Height cm	Maximal static force (MVC) (kp 90 extension)
PB	23	75	189	195
SG	22	62	171	205
SJ	21	85	193	161

TABLE II Individual data in two subjects for muscle concentration of ATP CP and lactate before during and after a sustained static contraction at 10 % of the MVC (see Table I) The contraction was interrupted for a few seconds to take the biopsy the same time as the 50 (I) and 25 % (II) tension was terminated due to exhaustion

	Rest			I				II				Exhaustion			
Subj	ATP *	CP*	Lactate*	Time	ATP *	CP*	Lactate*	Time min	ATP *	CP*	Lactate*	Time min	ATP *	CP*	Lactate*
SJ	4 7	14 5	1 3	2 67	4 5	16 6	2 1	7 98	5 6	11 5	2 2	36 00	5 1	10 5	4 1
PB	4 4	12 7	1 3	1 13	4 9	12 0	2 5	5 35	5 4	10 9	4 1	45 00	4 2	8 3	5 6

* mmoles kg⁻ wet muscle

The zero level was equal to the tension developed with the legs resting on the bar The bar strain gauge assembly was calibrated with adequate weights prior to the experiments Maximal voluntary contraction (MVC) for each subject was determined repeatedly in preliminary experiments a few days prior to the studies The final experiments were performed until exhaustion at 75 50 25 and 10 % of the MCV on separate days In additional experiments two of the subjects were studied while maintaining the lowest force for times corresponding to those attained at 50 and 25 % of the MVC (see Table II) The experiments were conducted over a 3 week period

Biopsies for the determination of muscle metabolites were taken from the lateral parts of the *quadriceps femoris muscle before (in supine position) and after (within 3—5 s) the exercise* with the subject still sitting in the chair The specimens were immediately frozen in liquid nitrogen and stored at -70 until analyzed for glycogen lactate ATP and CP as described earlier (Karlsson 1971) Blood samples were simultaneously taken from a prewarmed hand (fingertip blood) and analyzed for lactate concentration according to Scholz *et al* (1959)

Results

The pattern of maximal endurance time and relative force was similar to that reported by Monod and Scherrer (1957) and later by Rohmert (1960) Thus maximal endurance time increased rapidly when the relative force was less than 20—25 % (Fig 1) Phosphagen depletion (ATP and CP) was more pronounced with the 50 and 25 % tensions This was primarily produced by CP depletion with only a small reduction in ATP stores (Fig 2) This is in contrast to what is observed during bicycle exercise where a CP depletion to about 2 mmol×kg⁻¹ wet muscle (as the case with the 50 % tension) is acompanied by a significant ATP depletion (Karlsson Diamant and Saltin 1970 Karlsson 1971)

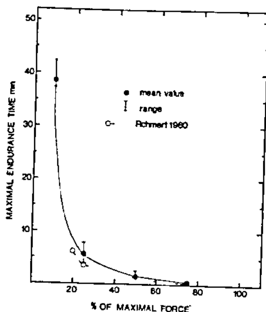


Fig 1 Maximal endurance time for static muscular contraction in relation to percent of maximal static force (MVC)

The pattern of muscle lactate concentration with isometric exercise was similar to that of CP depletion (Fig 2) The highest average concentration (21 8 mmol × kg⁻¹ wet muscle) was found with the 50 % tension exhaustion time Lactate concentration of the thigh was lower in all 3 subjects both at higher (75 % 0 5 min duration) and lower (25 % 5 8 min duration) relative tensions (average value 10 3 14 0 and 4 0 mmol × kg⁻¹ wet muscle with 75 25 and 10 % respectively of MVC)

Blood lactate concentrations immediately at the end of the exercise did not exceed 6—7 mmol × l⁻¹ (Fig 2) and were 2—3 time lower than in the muscle at all intensities Blood lactate concentrations were similar at rest and at the lowest load of tension Even though blood lactate concentrations were low they changed in a pattern similar to that of muscle lactate At tensions corresponding to 10 % MVC maintained for times that produced exhaustion at 50 and 25 % of MVC a gradual decrease in CP concentration and increase in muscle lactate concentration occurred (Table II)

The glycogen depletion averaged 27 25 33 and 16 mmol glucose units × kg⁻¹ wet muscle with 75 50 25 and 10 % respectively of the individual maximal static force However a considerable variation existed both intra and interindividually The inconsistent picture for glycogen depletion may be attributed to the fact that the methodological error is larger for the glycogen than the lactate determination (Karlsson 1971) One glucose residue produces 2 molecules of lactate during anaerobic glycolysis which will enhance the uncertainty when relating lactate formation to glycogen depletion

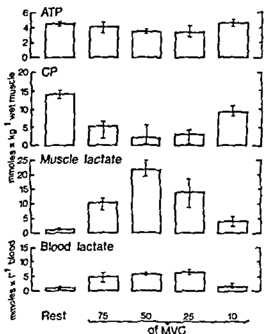


Fig 2

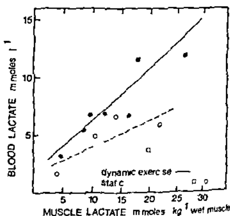


Fig 3

Fig 2 Mean values and range for ATP CP muscle and blood lactate concentrations at rest and at exhaustion in the different experiments with different percents of MVC (see Fig 1)

Fig 3 The relation between simultaneously obtained muscle and blood lactate concentrations for static and dynamic exercise In the figure are included the present mean values (o) data from one subject (■ □) studied at different dynamic and static exercise loads to exhaustion (unpublished results) and data from Karlsson and Saltin (1970) (■)

Discussion

From the phosphagen depletion and the lactate concentration data it could be concluded that the largest anaerobic output occurred when 50 % of the MVC was sustained for 1 6 min With this tension the phosphagen depletion and lactate concentration in the contracting muscle was similar to maximal values obtained during short term maximal bicycle work (Karlsson and Saltin 1970 Karlsson 1971) At the other test tensions lower values were obtained for the phosphagen depletion and lactate concentration in the muscle

In a recent study by Bergstrom *et al* (1971) muscle metabolism was examined in relation to isometric exercise but with a somewhat different type of leg extension contraction Bergstrom *et al* found that the ATP depletion at the two tensions studied, maximal and 40 % of MVC respectively were small compared to what usually was observed with dynamic exercise in relation to a corresponding decrease for CP concentration Moreover they demonstrated a more pronounced CP depletion and lactate accumulation with the 40 % tension compared to the maximal

No values were obtained concerning the aerobic energy output during the static

exercise The oxygen delivery to the contracting muscle is depending on the magnitude of the blood flow and to what extent it is nutritive Barcroft and Miller (1939) reported that in the calf muscle the blood flow might be occluded at isometric contractions corresponding to more than 20 % of MVC thus with higher tensions limiting the aerobic energy output to the amount of molecular oxygen present in the muscle at the start of contraction Humphreys and Lind (1963) have modified this concept by demonstrating an increased blood flow compared to resting levels in the forearm at as high tensions as 60 % of MVC but with a very pronounced post exercise hyperaemia (Lind *et al* 1964) indicating that the existing blood flow was far from adequate A restricted blood supply might affect the muscle tissue in many respects *e g* as already emphasized an inadequate oxygen delivery a diminished outflow of anaerobic metabolites and a reduced conductance of heat away from the contracting muscle The steeper concentration gradient between blood lactate and muscle lactate after static compared to dynamic exercise supports the suggestion of a restricted blood flow through the muscle during static contractions

Both heat accumulation and lactate concentration have been suggested as being closely related to the experience of muscular fatigue during bicycle exercise of 2—20 min duration (Karlsson 1971) Akre and Aukland (1970) have studied the heat generation in human forearm muscle during maintained as well as rhythmic isometric contractions and found it to be related to relative tension but according to their findings the calculated muscle temperature increase in the present study might only be 1—2° C which is less than found during exhaustive bicycle exercise of 2—20 min duration (Saltin *et al* 1972) The role of temperature as a main limiting factor might then be excluded of these reasons

The low blood lactate versus the high muscle lactate concentrations might indicate a higher gradient between muscle and blood for static as compared to dynamic exercise (Fig 3) The most reasonable explanation for this seems to be the occlusive effect on the capillary bed with sustained static tensions causing a delayed efflux as already discussed above

Another indication that an aerobic energy yield was absent or very small when pushing with 50 and 75 % MVC is that in these 2 expts the product of tension × time is very similar and much less than in the experiments with 10 and 20 % of MVC It may be worth mentioning that according to Hoes *et al* (1968) only up to 15 % of MVC is used during maximal bicycle work which can be sustained for 4—6 min

It has been mentioned that the highest anaerobic energy output in terms of phosphagen depletion and lactate formation was found with the 50 % tensions The reason why lower muscle lactate concentrations and phosphagen depletions were obtained with the other tensions cannot fully be explained at present Different recruitment patterns of muscle fibers with different contraction tensions might limit the phosphagen depletion and the lactate accumulation to only the fibers recruited Local factors within these fibers would then limit the performance of all the muscle The values for phosphagen depletion and lactate accumulation obtained in a biopsy

specimen repre ent a number of fibers Differences in ATP CP as well as lactate concentration within the analyzed specimen might then be expected However, additional factors cannot be excluded as of importance to the experience of muscular fatigue at those tensions where maximal phosphagen depletions and lactate accumulations were not observed

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Effect of Sympathetic and Parasympathetic Stimulation on the Secretion and Outflow of Aqueous Humour in the Rabbit Eye

By

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Abstract

Uusitalo R *Effect of sympathetic and parasympathetic stimulation on the secretion and outflow of aqueous humour in the rabbit eye* Acta physiol scand 1972 *86* 315—326

The influence of electrical stimulation of the superior cervical sympathetic ganglion or parasympathetic (oculomotor) preganglionic nerves on the IOP and intraocular fluid dynamics of rabbits was investigated Special attention was paid to the inflow of aqueous humour and the NaK ATPase activity in the ciliary body iris after these stimulations Sustained sympathetic stimulation was found to cause a significant decrease in the equilibrium pressure of the eye This stimulation was found to decrease the rate of secretion of aqueous humour but not to influence significantly the rate of drainage of aqueous humour Parasympathetic stimulation caused a significant increase in IOP which was due to an increased inflow of aqueous humour The NaK ATPase activity measured after these stimulations in the ciliary body iris was unaffected

Key words Sympathetic stimulation parasympathetic stimulation intraocular pressure adenosine triphosphatase ciliary body iris

The level of intraocular pressure (IOP) is regulated mainly by the inflow rate and outflow facility of the aqueous humour (Sears 1960) The nature of the mechanism regulating secretion of aqueous humour which takes place in the ciliary epithelium is relatively unknown (Kinsey and Reddy 1964) On the other hand much more information has been published on the regulation of the outflow (Eakins and Ryan 1964)

The rich autonomic innervation of the eye has tempted many workers to make a correlation between the intraocular fluid dynamics and the innervations (Sears 1966) The main interest has been in the correlation between the sympathetic nerve system and the outflow mechanism (Sears and Gillis 1967 Rosser and Sears 1968) Generally speaking sympathetic denervation causes a transient increase in the outflow which thus causes a transient decrease in intraocular pressure (Langham and Taylor 1959 1960 a b Sears and Barány 1960)

The effect of parasympathetic denervation on the intraocular fluid dynamics has been studied very little One of the most comprehensive works was by Schmerl and Steinberg (1949) who claimed that coagulation of the ciliary ganglion caused a decrease in intraocular pressure and this recovered to the original pressure level after 2—4 weeks

Stimulation of the sympathetic nervous system has been performed by many authors whereas very little attention has been paid to the effect of stimulation of the parasympathetic system It has been commonly agreed that stimulation of the sympathetic system decreased the intraocular pressure (Langham and Rosenthal 1966)

When the parasympathetic ganglion has been stimulated a clear increase or decrease in intraocular pressure has been observed (Schmerl and Steinberg 1949 Armaly 1959)

The literature clearly indicates considerable confusion as to what actually happens in the eye when either the sympathetic or the parasympathetic nerves are denervated or stimulated Many papers have been particularly concerned with intraocular pressure inflow or outflow or two of these factors but very seldom with the whole triangle In my opinion all three factors must be considered The question is still more complicated when the effects of vascular changes obviously present in connection with the changed nervous control of the eye are taken into account (Cole and Rumble 1970)

This study is a continuation of our earlier work in which we described the effect of sympathetic and parasympathetic denervation on the inflow rate and outflow facility of the aqueous humour and its effect on intraocular pressure (Harkonen *et al* 1972)

The purpose of the present work was to study how ocular fluid dynamics change through stimulation of the autonomic nerve systems (either sympathetic or parasympathetic) Special attention has been paid to the pump enzyme or NaK ATPase activity which seems to play an outstanding role in the secretion of aqueous humour (Bonting and Becker 1964 Palkama and Uusitalo 1969)

Material and methods

The material consisted of 20 male albino rabbits weighing 1–3 kg The animals were anesthetized with urethane (1 0–1 75 g kg) or thiopentone sodium (0 025–0 030 g/kg) diluted in distilled water and injected into a marginal ear vein

The IOP was recorded manometrically using a pressure transducer (Swema SP 23 D) together with a recorder (Metrohm E 478) The technique used was a modification of the method described by Sears and Barany (1960) It has also been described in more detail by Harkonen *et al* (1972) Tetracain 0 5 Star was instilled into the eyes before inserting the needles If local anesthesia was not used a rapid rise in IOP was seen

The manometric system was filled with isotonic saline At the moment when the needle was introduced into the anterior chamber a reservoir connected to the manometer was adjusted to give a pressure of about 25 mm Hg The needle with the opening in its shaft was carefully placed so that it did not touch the iris The needle was closed off from the reservoir as soon as properly located After cannulation, IOP was allowed to stabilize for at least 20 min in order to get preinfusion pressure (P_0) The facility of aqueous outflow (C) was measured by noting the pressure change (ΔP) resulting from a constant rate anterior chamber infusion of isotonic saline The infusion rate (ΔF) was adjusted to be about 2 to 3 µl min which caused an increased IOP of about 6 to 8 mm Hg The only experiments accepted were those in which a clear 5 min plateau was reached during infusion. This new equilibrium P pressure was generally reached within 15 min of the start of infusion. When infusion was stopped the pressure reached the postinfusion level (P) usually within 15 min If it differed more than 1 mm Hg from the preinfusion pressure the experiment was discarded

In calculating the inflow rate and outflow facility it was assumed that no change in aqueous secretion occurred due to a changed IOP The importance of this has been described in detail by Sears (1960) Facility calculations were made according to the formula $C = \Delta F/\Delta P$ where ΔF equals the particular infusion rate Values for aqueous inflow were derived from the formula $F = C$

(P − P) where the P (episcleral pressure) was assumed to be constant at 9 mm Hg (Kornbluth and Linner 1955)

Sympathetic stim lation A midline incision was made and the sternohyoid and omohyoid muscles separated to expose the carotid artery the right vagus nerve and the superior cervical sympathetic ganglion A stainless steel electrod was introduced into the ganglion A drop of histoacryl N (Braun Melsungen) was inst lled on the steel electrode to fix it to the ganglion The uninsulated tip of the monopolar electrode was 2 mm in length and 0 5 mm thick During stimulation this electrode was cathodal with respect to the indifferent remote electrode inserted subcutaneously

After the operation and insertion of the electrode the skin was sutured and the rabbits were tied prone to a board in a comfortable position and placed in a head holder This was done to avoid changes in th eye level w th respect to the transducer Before the stimulation period was started the ocular te sion inflo rate and outflow facility were always measured from the control (l ft) eye Thereafter the experimental eye (right) was cannulated and the original (prestimulation) pressure (P) was recorded

Unidirectional square wave stimuli were then applied with a stimulator having a constant current output Stimulation was carried out by applying pulses at the rate of 13 pulses per sec dura tion 2 5 msec each and current output about 0 6 mA The stimulation caused an immediate de c ease in intraocular pressure about 4 mm Hg below the original pressure le el (Table I) Equi libration was attained within 15 min of stimulation and prolonged stimulation up to 60 min did not cause any add tional change in pressure Once equilibration was reached during sympathetic stimulation, infusion was started to measure inflow rate and outflow facility

Pa asymp thetic st mulation Preganglionic oculomotor nerve stimulation v as carried out according to a stereotaxic technique Through a frontal burr hole situated on the coronal suture or slightly anterior to it 1 8 mm lateral to the sag ttal suture a b polar teflon coated steel electrode (Leico Industries) was inserted into the target area (Monnier and Gangloff 1961) The bipolar electrodes were made from 2 steel wires cemented together with araldite The target area was where the oculomotor nerve leaves the brain This was s tuated 20 mm below the upper surface of the skull The uninsulated tip of the bipolar electrode was 0 5 mm in length and 0 13 mm thick The surface area of the tip was approximately 0 11 mm and the interelectrode distance 2 mm When the electrode was n the right position a marked immed ate m osis was observed as a response to stimula tion In a ser es of rabb ts the locat on of the electrode was checked by producing an electro-coag lative lesion with the same electrode by means of a h gh frequency coagulator (Martin Elektrotom 25) Th unidirectional square wave stimuli were appl ed by means of a st mulator with a constant current output The current through and the voltage drop across most of the electrical rabb ts was mon tored in order to obtain information about the stimulus parameters and the electrical beha viour of the electrode (Johansson 1969) Stimulation was carried out by applying pulses w th repe tition frequencies of 44 pulses per sec duration 0 8 msec and current output about 2−3 mA

Measurements of intr ocular fluid dynamics were carried out s pre iously described in connection with sympathetic stimulation The IOP inflow rate and outflow facil ty were first measured in the con trol (left) eye Then the experimental (right) eye was cannulated and the o iginal (prestimulation) pressure (P) was measured An immediate increase in IOP follov ed by a slow reco ery to a level about 4−5 mm Hg above the prestimulat on pressu e was recorded during parasympathetic stim ulat n Equilibration was attained with n 15 to 20 min Prolong d st mulation to 60 m n did not cause any additional pressure change When equ l brium was eached infus on was started to m asure inflow rat and outflow facility

P a ympathet c st mulation after superior cervic l g glio ectomy In order to eliminate the sympathetic component during parasympathetic stimulation 3 alb no rabb ts underwent super or cervical ganglionectemy under th openton sod um anesth sia 7 days befor parasympathet stimulat on The preganglionic parasympathetic stimulat on was carried out as de cribed above

M asurement of pup l diameter Measurements of pupil size were made w th a m ll meter ruler viewed at a distance of 20 cm from the cornea The exp riments wer always carried out n ord nary laboratory l ght ng The pup l diameters of the experimental and control eyes wer recorded just bef re the beg nning of stimulation after the stimulation had been turn d on just before it was turn d off and immed ately aft it ended

ATPase measurements Following the st mulations and pressure measurements the animals were killed by an i v air injection The two ey s were imm d ately enucleated and opened anter o ly the lens and the vit eous were r moved and each ciliary body ris prepr rat on was exci ed as a complete ring The block w re ut into two hal es immed ately immersed in liquid n t og n and stored at −50 C The l ochemical N K ATPase analyses were made fluorom trically as d scribed previously (Harkonen *et al* 1972) The rout ne assay proc dure was the same as that de scribed ea lier except that amytal was excluded due to its inhib tory eff ct on NaK ATPase (Jarne felt 1962 Harkonen *e l* 1972) Homogenates of whole c l ary body iri were incubate l at 37 C in 1 ml of reagent cont in ng 50 mM Tr s-HCl buffe pH 8 1 1 9 mM MgCl 0 5 mM PEP 1 6 mM ATP 0 05 mM NADH and 00 2 BSA When total ATPase was measured the media con

TABLE I Effect of sympathetic and parasympathetic stimulation on intraocular pressure (IOP) observed at different time intervals during the experiment

Sympathetic Stimulation			Parasympathetic Stimulation		
Stimulation Time	Number of Eyes	IOP mm Hg	Stimulation Time	Number of Eyes	IOP mm Hg
0	8	16.5 ± 1.0	0	9	18.6 ± 0.8
15 sec	8	13.5 ± 0.6	–	–	–
30 sec	8	13.1 ± 0.6	–	–	–
45 sec	8	13.5 ± 0.7	–	–	–
1 min	8	13.0 ± 0.6	1 min	9	29.4 ± 1.5
2 min	8	12.6 ± 0.6	2 min	9	26.7 ± 1.0
5 min	8	12.9 ± 0.7	5 min	9	23.7 ± 0.8
10 min	8	13.1 ± 0.7	10 min	9	23.9 ± 1.0
15 min	8	13.1 ± 0.6	15 min	9	23.5 ± 1.2

Standard errors are those of the mean

tained 10 mM Na and 30 mM K. When measuring Mg ATPase Na and K were omitted. As NaK ATPase activity cannot be determined directly in a crude preparation such as that used in this study, net NaK ATPase was calculated from the difference between total ATPase and Mg ATPase.

Statistical evaluation In the preliminary studies it was soon found that inflow rate and outflow facility could be measured reliably only once. If more than one measurement was made in the same eye the variation was greater than it was between the right and left eyes of the same animal. The right (experimental) eye was always compared to its fellow eye and the results were analysed using the matched pair t test (Richterich 1968). This test reveals whether the difference between the two fellow eyes differs significantly from zero.

The ATPase activities in the ciliary body iris were also statistically analysed according to the matched pair t test.

Results

Stimulation parameters In the preliminary studies an attempt was made to discover the maximal effect of stimulation by varying the parameters of stimulation. The maximal effect of sympathetic stimulation on IOP was obtained using 10 to 20 stimuli per sec. In the later studies a rate of 13 stimuli per sec was chosen. Using 20 stimuli per sec did not increase the effects. According to the present observations maximal iris contraction and the clearest change in IOP during parasympathetic stimulation in the rabbit was reached when the number of stimuli was increased from 20 to 50 stimuli per sec and thus a rate of 44 per sec was used.

Reliability of prolonged stimulation In all experiments involving stimulation of the cervical sympathetic ganglion (characteristics of the stimulation are 0.6 mA, 2.5 msec, 13 per sec) the pupil on the stimulated side was either maximally dilated or at least obviously more dilated than the pupil of the unstimulated control eye. Continuous stimulation prolonged for 20 to 25 min did not result in any relaxation of the dilatator muscle of the iris.

Prolonged stimulation of the preganglionic parasympathetic fibers (2–3 mA, 0.8 msec and 44 pulses per sec) resulted in a contraction of the sphincter of the iris. The pupil diameter decreased from 6 to about 3 mm. With these stimulation parameters fatigue was not observed during 30 min of stimulation.

TABLE II Effect of sympathetic and parasympathetic stimulation on intraocular pressure (IOP) outflow facility (C) and inflow (F)

Type of Stimulation	Number of Eyes	IOP mm Hg	C µl/min/mm Hg	F µl/min
Sympathetic Stimulation				
Control (Left) Eyes	6	18.0±1.1	0.31±0.05	2.56±0.25
Experimental (Right) Eyes	6	13.2±0.7	0.22±0.03	0.81±0.16
Mean Difference		4.7±0.7	0.09±0.03*	1.75±0.20
Parasympathetic Stimulation				
Control (Left) Eyes	9	17.9±0.4	0.21±0.02	1.86±0.18
Experimental (Right) Eyes	9	23.3±1.1	0.45±0.08	6.66±1.49
Mean Difference		−5.4±0.9	−0.23±0.09	−4.80±1.53

The control eyes were measured before stimulation was started. The statistical significance between the paired eyes (matched pair t test) is marked * if $P < 0.05$, ** if $P < 0.01$ and *** if $P < 0.001$. Standard errors are those of the mean.

Intraocular pressure during electrical stimulation of the sympathetic or parasympathetic nerve

Table I summarizes the effect on IOP of varying the period of electrical stimulation of the sympathetic or parasympathetic nerve. During sympathetic stimulation there was an immediate decrease in IOP to a pressure about 4–5 mm Hg below prestimulation level (P_0) in all the eyes. Equilibration was attained within 5–10 min after the beginning of stimulation. Further stimulation even for 60 min did not cause any additional change. The experimental results of the effects of electrical stimulation of parasympathetic nerves on IOP are shown in Table I. There was an immediate increase in IOP of approximately 10–12 mm Hg followed by a slow recovery to a pressure level about 5–6 mmHg above the prestimulation pressure. Equilibration was attained as during sympathetic stimulation within 5–10 min and was unaffected by prolonged stimulation up to 60 min.

Changes in aqueous humour dynamics during sympathetic or parasympathetic stimulation

The manometric findings during stimulation of the sympathetic or parasympathetic nerve are shown in Table II. The results reveal that during stimulation of the superior cervical ganglion there was a marked decrease in IOP ($P>0.01$) and a decrease in the rate of secretion of aqueous humour ($P<0.001$) compared with the paired control eye. In the calculations episcleral pressure (P_v) was assumed to stay constant at 9 mm Hg. It was also noted that stimulation of the cervical ganglion caused a decrease in outflow facility ($P<0.05$).

During stimulation of parasympathetic preganglionic nerves IOP increased more than 5 mmHg in the ipsilateral eye ($P<0.001$). The increase in IOP was due to the increased inflow assuming that P_v stayed constant at 9 mm Hg. The inflow rate was about three times that of the unstimulated (contralateral) eye ($P<0.05$). During this parasympathetic stimulation outflow facility was also slightly increased but this change was not significant ($P>0.05$).

TABLE III Effect of sympathetic and parasympathetic stimulation on ATPase activity in the ciliary body iris

Type of Stimulation	Number of Eyes	Total ATPase	Mg ATPase 10[illegible] μmol/mg prot/min	NaK ATPase
Sympathetic Stimulation				
Control (Left) Eyes	8	5.40±0.38	4.02±0.36	1.38±0.19
Experimental (Right) Eyes	8	5.08±0.51	4.16±0.58	1.09±0.23
Mean Difference		0.33±0.36	−0.13±0.5[illegible]	0.29±0.[illegible]1
Parasympathetic Stimulation				
Control (Left) Eyes	9	7.45±0.48	6.2[illegible]±0.57	1.23±0.19
Experimental (Right) Eyes	9	6.76±0.84	5.66±0.57	1.17±0.40
Mean Difference		0.69±0.61	0.56±0.64	0.06±0.47

Statistical analyses revealed no significant differences between paired eyes Standard errors are those of the mean

When superior cervical ganglionectomy was made 7 days before ipsilateral parasympathetic stimulation the increases in IOP and inflow were of the same degree as in animals that had not been subjected to ganglionectomy

Effect of sympathetic or parasympathetic stimulation on ATPase activities in the ciliary body iris

After neither sympathetic nor parasympathetic stimulation for 30 min was there any significant change in Mg ATPase or in NaK ATPase activity in the experimental eyes (Table III)

When cervical sympathectomy was made 7 days before parasympathetic stimulation the results of ATPase activities were about the same as in the parasympathetic group

Discussion

The stimulation parameters used in the present study were found to be in close agreement with those of others According to Folkow (1952) the upper limit for the physiological discharge rate in the vasoconstrictor (sympathetic) nerve is 6 to 8 stimuli per sec Maximal response was obtained with 10 to 15 stimuli per sec In the present study sympathetic nerves were stimulated by using a frequency of 15 per sec This gave a good response (mydriasis and decrease in IOP) and was used throughout the experiments

The physiological discharge rate in the parasympathetic system has been claimed to be somewhat higher than in the sympathetic system (Luco and Salvestrini 1942) They obtained a maximal effect for iris contraction in the cat using 25 stimuli per sec According to the present observations maximal miosis was observed when the stimuli numbered 44 per sec

These experimental results indicate that electrical stimulation of the cervical superior ganglion decreased the rate of secretion of aqueous humour and the equili

drum pressure of the eye (Table II) On the other hand stimulation of the ganglion did not significantly change the outflow facility of the eye although this was slightly decreased (Table II)

Many investigators have shown that stimulation of the cervical sympathetic nerve in rabbits causes a lowering of the intraocular pressure (Henderson and Starling 1904 Wessely 1908 Davson and Matchett 1951 Greaves and Perkins 1952) Langham and Rosenthal (1966) prolonged the stimulation time which according to them was sufficiently long to distinguish the pressure change due to vasoconstriction from that due to an alteration in the intraocular fluid dynamics They found a decreased IOP and secretion of aqueous humour The long stimulation (30 min) applied in the present study provides evidence in support of the claim that sympathetic stimulation causes an alteration in the intraocular fluid dynamics Table II)

The decrease in IOP during sympathetic stimulation from an average value of 18 0 mm Hg to a new steady state value of 13 2 mm Hg is a significant change This decreased IOP reflects a marked change either in the rate of aqueous humour formation or the outflow facility, or the venous pressure opposing the outflow of the aqueous humour It is generally accepted that the dynamics of the aqueous circulation are described by the relationship $F = C(P_0 - P_v)$ (see material and methods Thus the fall in IOP of 4 7 mm Hg observed in the present studies could be accounted for by a decrease of approximately 40 % in either the rate of aqueous inflow or the resistance of outflow (I/C) or by a decrease of 4 7 mm Hg in the venous pressure \The present studies indicate that the fall in the equilibrium pressure of the eye following sympathetic stimulation was not due to a change in the outflow but was the result of a decrease in inflow assuming that P_v stayed at 9 mm Hg These results are in good accordance with the findings of Langham and Rosenthal (1966) who reported that during sympathetic stimulation the rate of inflow decreased by about 30 per cent They derived the rate of aqueous humour formation from studies of turnover of fluorescein in the aqueous humour

The possibility that simultaneously with the decreased inflow sympathetic stimulation caused a decrease in venous pressure cannot be excluded on the basis of this study However this is not very likely according to the results reported by Langham and Rosenthal (1966) They claimed that if the P_v is changed during sympathetic stimulation this effect is evidently of minor importance to the decreased IOP

Reduced blood flow in the eye capillaries during sympathetic stimulation seems to play a major role in explaining the decreased inflow and thus also the changed IOP This point of view is supported by the reports of Linner (1952) and Langham (1955) They estimated the rate of blood flow to the ciliary processes during electrical stimulation of preganglionic cervical sympathetic nerves by injecting ascorbic acid into the vascular system and using it as an indicator of blood flow The results of ascorbic acid analyses indicated that stimulation caused a 50 per cent decrease of the blood flow into the ciliary processes This means that the decrease in the rate of inflow could be due primarily to a reduced blood flow and secondly to direct inhibition of the secretory mechanism The major importance of the reduced

blood flow to the decreased inflow during sympathetic stimulation is not, however quite clear It has been reported that a decreased blood flow of 20—40 % to the eye induced by unilateral carotid ligation failed to decrease inflow (Bárány 1947 Davson and Matchett 1951 Linner 1952, Langham 1955)

Evidence of specific inhibition of the secretory mechanism by sympathomimetic drugs acting on *in vitro* preparations of ciliary processes has been found by Berggren (1965) In the present work it was found that the activity of NaK ATPase in the ciliary body iris was not significantly decreased after 30 min of stimulation of the sympathetic nerves This means that the decreased inflow was not caused by the part of the secretory mechanism that remained uninfluenced by stimulated sympathetic nerves. Although the present studies as presented favour the opinion that decreased secretion could have been secondary to reduced blood flow one cannot exclude the possibility of a direct inhibition of aqueous humour secretion by the sympathetic transmitter caused by stimulation of the cervical ganglion The decreased inflow could possibly reflect an inhibition due to some active transport mechanism other than the NaK ATPase system

In the present study it was also found that sympathetic stimulation caused a slight decrease in outflow facility ($P<0.05$) Langham and Rosenthal (1966) similarly found that sympathetic stimulation resulted in a decrease in IOP without any significant effect on outflow facility Casey (1966) who studied monkeys found that after stimulation of the cervical sympathetic ganglion there appeared in the homolateral eye a moderate but statistically significant decrease in outflow facility These findings are contradictory to the claim that adrenergic receptors increase outflow facility (Langham and Taylor 1959 1960 a b, Sears and Barány 1960 Barány 1962 Eakins and Eakins 1964 Paterson 1966)

Sears and Barany (1960) showed that excision of the superior cervical ganglion in the rabbit causes a decrease in IOP due to an increase in the outflow facility of aqueous humour They explained the phenomenon as being partly due to the release of a facility increasing alpha adrenergic substance from degenerating nerve endings in the ciliary body and iris Their interpretation was based on tests in which intravenous injection of the alpha adrenergic antagonist dibenamine, decreased outflow facility However subsequent studies with other more specific adrenergic antagonist chemicals have failed to confirm this claim (Langham 1955 Bárány 1962 Eakins and Ryan 1964) Paterson (1966) reported that sympathetic stimulation caused a clear decrease in IOP simultaneous with a non significant increase in outflow facility This discrepancy between the present findings and those of Paterson may be due to many factors *e g* the type of narcosis However we must note that in both works the change in outflow was non significant and thus of minor importance Furthermore we must also remember the possibility that the alpha adrenergic receptors present in the outflow mechanism are not innervated at all by sympathetic fibers

The earlier literature concerning the effect of the parasympathetic system on ocular fluid dynamics is indefinite Schmerl and Steinberg (1949) stimulated the ciliary ganglion of rabbits by introducing bipolar electrode through an incision in the

anterior lower part of the eyelid They observed an immediate increase in IOP which changed from 8 to 17 mm Hg Perkins (1954) stimulated the third cranial nerve in rabbits and produced a rise in IOP This was however possibly due to a contraction of the extraocular muscles and could be abolished by paralysing the extraocular muscles with decamethonium iodide (Greaves and Perkins 1952) However, they did not prolong the stimulation sufficiently to distinguish whether the pressure change was due to contraction of the extraocular muscles or to an alteration in the intraocular fluid dynamics The prolongation of stimulation (30 min) in the present study gives the first evidence that parasympathetic stimulation does cause a significant increase in the equilibrium pressure of the eye and consequently an alteration in the intraocular fluid dynamics The increase in IOP from an average value of 17 9 to a new steady state value of 23 3 mm Hg (difference 5 4 mm Hg) was highly significant

Is this increased IOP due to an increase in aqueous humour formation changed outflow facility or venous pressure? The present manometric studies indicate that the increase in IOP following parasympathetic stimulation was due to an increase in inflow assuming that P_V stayed normal The outflow facility was not changed Whether parasympathetic stimulation caused an increase in the episcleral pressure could not be determined since the P_V was not measured during stimulations

The marked increase in the rate of secretion of aqueous humour during parasympathetic stimulation raises the question of whether the increased secretion was secondary to increased blood flow or due to some other change in the secretory mechanism Whether the parasympathetic stimulation really increased the blood flow cannot be answered There have been some investigations dealing with the effects of parasympathetic nerves on blood flow in the eye Colle *et al* (1931) working with dogs reported an increase in IOP after administration of acetylcholine (ACh) to the uveal vessels This was considered to be due to the vasodilatatory effect of the drug Sallmann (1955) electrically stimulated the diencephalon of the cat in the parasympathetic area He found that stimulation in this area resulted in either elevations or falls of IOP These were considered not to be connected either with changes in the systemic blood pressure or tension of extraocular muscles According to Sallmann this points to local centrally elicited changes in the functional state of the intraocular vasculature Bill (1962) demonstrated that ACh reduces the vascular resistance controlling the uveal blood flow The author found furthermore that stimulation of the cholinergic fibers of the uvea in cats did not produce vasodilatation and proposed that ACh normally liberated within the eye does not reach the uveal vessels sensitive to it Perkins (1957) also reports that stimulation of the third cranial nerve of rabbits does not cause vasodilatation in the uvea

In the light of the above studies it is probable that in the present study too stimulation of the parasympathetic nerve did not cause an increase in blood flow into the eye This favours the view that stimulation had an effect on the active transport mechanism It is also possible that parasympathetic stimulation could increase the permeability of the blood aqueous barrier without causing vasodilatation

A digitalis sensitive sodium potassium activated adenosinetriphosphatase system (NaK ATPase) which has been implied in active cation transport (Skou 1957 1960 Post *et al* 1960 Dunham and Glynn 1961 Bonting *et al* 1962 1963 1964) has been demonstrated in the ciliary epithelium and shown to act in the secretion of the aqueous humour into the eye (Bonting *et al* 1961) In the present investigation the increased pressure during parasympathetic stimulation was obviously due to an increase in the rate of inflow of the aqueous humour assuming that episcleral pressure remained constant According to earlier reports by Uusitalo and Palkama (1971) parasympathetic denervation decreased NaK ATPase activity in the ciliary body iris Thus it was of special interest to see whether any correlation could be noted between parasympathetic stimulation and NaK ATPase activity in the ciliary body iris The activities of Mg ATPase and NaK ATPase were measured in the ciliary body iris after 30 min of stimulation of the parasympathetic nerves However, there was no observable difference in enzyme activity between the stimulated and unstimulated eye This means that at least this part of the active transport system is not influenced by stimulation of the parasympathetic nerves such as used in the present experiments These findings do not exclude a possible relationship between parasympathetic stimulation and NaK ATPase activity in the ciliary body iris It may be that parasympathetic impulses are necessary to NaK ATPase function but that beyond a certain threshold of nerve activity the enzyme response remains unchanged On the other hand if this threshold is not reached (after denervation) the enzyme activity decreases and with it the inflow of aqueous humour and the IOP

The effect of parasympathetic nerves on the mechanism regulating the IOP needs further investigation It has however been shown that parasympathetic stimulation causes a significant increase in the (equilibrium) pressure of the eye Whether this effect is based on changes purely in the vascular or in the secretory mechanism cannot yet be determined It may be that there are several functions regulating the inflow of aqueous humour which are under nervous control

In conclusion it can be noted that the present work supports the earlier reports by our group according to which ocular tension is under nervous control (Uusitalo and Palkama 1971)

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Functional Dependence of the Ciliary Epithelium ATPase Activity and Intraocular Pressure on the Autonomic Nervous System

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Abstract

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Regulation of intraocular pressure by autonomic nerves of the eye was studied using three types of denervation sympathectomy ciliarectomy or coagulation of the parasympathetic nerves (around the optic nerve) Intraocular pressure inflow and outflow were registered manometrically using a constant pressure infusion technique ATPase activities in the ciliary processes were demonstrated histochemically and assayed fluorometrically Special attention was paid to the biochemical characteristics of the ciliary body NaK ATPase activity A kinetic fluorometric method for NaK ATPase was developed Intraocular pressure decreased after ciliarectomy simultaneously with a decrease in inflow of the aqueous humour Ciliary body iris NaK ATPase activity also decreased The changes in the ocular fluid dynamics after coagulation of the parasympathetic nerves tended to be the same Intraocular pressure decreased as well as NaK ATPase activity Sympathectomy however did not change ocular fluid dynamics or NaK ATPase activity The ciliary body iris NaK ATPase activity seemed to have the characteristics of the transport enzyme found in other organs It was activated by Na K and Mg and could be inhibited by ouabain Activity was optimum when the Mg/ATP ratio was about 1 and the optimal pH both for Mg and NaK ATPase was 8 1 The results give further support to the theory that intraocular pressure is under nervous control This regulation is mediated by decreased inflow which in turn is reflected by lowered NaK ATPase activity in the ciliary processes

Key words Adenosine triphosphatase ciliary body denervation intraocular pressure

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Intraocular pressure (IOP) is mainly dependent on the inflow rate and outflow facility of the aqueous humour It is still an open question how these two mechanisms are regulated Several workers have investigated the possibility that the relatively rich autonomic nervous system in the eye plays a part (Laties and Jacobowitz 1964 Pataky 1970) The nerves originate either from the superior cervical ganglion (sympathetic nerves) or from the ciliary ganglion (parasympathetic nerves) (Eranko *et al* 1967)

Aqueous humour flows out by the anterior chamber angle and in by the ciliary body Comparison shows that the ciliary body seems to contain a much richer nervous network than the anterior chamber angle (Ehinger 1966) Thus it has

assumed that the nervous system regulates the inflow more than the outflow (Štaflova 1969) Furthermore it has been demonstrated that the pump enzyme or NaK ATPase activity is also more active in the area of the ciliary body than in the angle of the anterior chamber (Palkama and Uusitalo 1970)

The possible nervous control of IOP has mostly been studied by dissecting the adrenergic fibers of the eye Sears and Bárány (1960) demonstrated that after sympathectomy the outflow changed temporarily and the IOP decreased Recently however it has been suggested that the parasympathetic system has a more marked effect on IOP than the sympathetic system and especially on inflow (Palkama and Uusitalo 1968) Parasympathetic denervation of the eye caused a significant decrease in IOP and in addition this decrease occurred simultaneously with the decrease in NaK ATPase activity in the ciliary body (Uusitalo and Palkama 1971) This decreased NaK ATPase activity has been supposed to decrease transduction of sodium into the posterior chamber of the eye and thus decrease IOP

In the present study we deal in detail with the ciliary body ATPase activity intraocular pressure and inflow rate and outflow facility after sympathetic and or parasympathetic denervations Special attention has been paid to the biochemical characteristics of the ciliary NaK ATPase function

Material and methods

In all 57 male albino rabbits weighing between 1 and 3 kg were used in the experiments The rabbits weighing bet een 1 and 3 kg were used in the experiments The rabbits were divided into 3 groups according to the denervations performed 1 *sympathectomy* 2 *ciliarectomy* and 3 *parasympathectomy* groups The right eye was denervated

During the operations the animals were anesthetized by i v injection of 25 urethane (1 0–1 75 g/kg) or thiopentone sodium (0 025–0 050 g/kg) The animals were killed 1 2 5 or 30 days after the operations Before the animals were killed with an i v air injection the intraocular pressure and inflow rate and outflow facility were measured Samples were then taken from both eyes for histochemical and biochemical analyses The animals that lived more than one day after the operation received a dose of 600 000 I U benzylpenicillinprocain

Denervation techniques Group 1 Sympathectomy was performed by dissecting the right superior cervical ganglion (Fig 1)

Group 2 Ciliarectomy was performed by cutting the posterior ciliary nerves attached to the optic nerve or near it just befor their penetration into the eye (Fig 1) After cantothomy the conjunctiva and tenon s capsule were opened and special care was taken to avoid bleeding from the large retrobulbar venous sinuses and from the vessels of the ocular muscles Sterility was carefully maintained especially when the ciliary nerves were dissected retrobulbarly in animals to be killed more than one day later

Some of the animals were sham operated but no differences in ATPase activity or intraocular fluid dynamics could be detected when compared with the contralateral control eye

Group 3 Parasympathectomy was performed by electrically coagulating the right oculomotor nerve and the fine parasympathetic fibres running around it intracranially (Fig 1) This group contained only rabbits weighing 2 5–3 0 kg in order to get as precise coagulation as possible The stereotaxic technique used was as described by Monnier and Gangloff (1961)

Through a frontal burr hole on the coronal suture or slightly anterior to it (1 3 mm) and 1 8 mm lateral to the sagittal suture a unipolar steel electrode was introduced into the target area The electrode was insulated so that the bare tip was 1 mm The target area was located about 20 mm below the top level of the skull bone This is the area where the oculomotor nerve leaves the brain and lies intracranially (Monnier and Gangloff 1961)

The right oculomotor nerve was always stimulated electrically before it was coagulated to check the position of the needle When the needle was in the right position a good response in the iris with a marked miosis was observed immediately following stimulation using repetition frequencies of 44 pulses per sec duration 0 8 msec and current output about 2–3 mA The coagulation was made at the same place

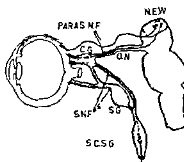


Fig 1

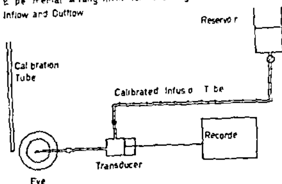


Fig 2

Fig 1 Schematic illustration of the innervation of the ciliary body Parasympathetic (Paras N F) and sympathetic nerve fibres (S N F) have been marked as ending in the ciliary body N E W = nucleus of Edinger Westphal S C S G = superior cervical sympathetic ganglion C G = cili ry gangl on S G = sympathetic ganglion O N = oculomotor nerve

Fig 2 Schematic drawing of the experimental arrangement for recording the IOP inflo v rate and outflow facility of the rabbit eye

The current used for coagulation was adjusted so that it caused a coagulation of egg proteins in an area about 1 5 m n in d ameter When there was no clear re ponse to stimulat on t o extra coagulat ons were made 0 5 mm to each s de of the orig nal point Only those rabbits in h ch total mydriasis was recorded and whose pupils did not react to l ght were accepted In son e cases mydria s s followed coagulat on immediately while the rabbits were still anesthetized but mydrias s usually followed one or two days afterwards (Fig 3) The animal in this group (3 parasy npa thetic group) were killed 5 days after the coagulation

After record ng the intraocular fluid dynamics the chol ne gic nerve fibres were demonst ated in both oculo notor nerve and c liary proces es of both eyes When a clear myd ias s as observed after the coagulation of the oculomotor nerve the coagulat on had been successful In some ase the left oculomotor nerve was also partially coagulated For this reason the left eye v as not accepted as a control eye in this group

P s u e measu ements Before killing the an mal the ocular tens on nflo and resi tance of outflo of the right (experimental) and left (control) eyes were measured manometrically in g oups 1 and 2 In group 3 the left eye was not accepted as a control eye In th s group the ght (e peri mental) eye v as compared with controls con ist ng of normal untreated eyes of other abbit These rabbits (9) v ere of the same age a id w ght as the experimental an mals The left (control) eyes of those animals which belonged to the other experimental groups were not accepted not only due to the differences in the age and we ght but also due to the poss ble effects of the oper ti n on the contralateral (left) s de (Eranko *et al* 1967)

During the mea urements the a umal we anesthetı ed with urethane and the r heads we fixed in a stereotax c frame All the an mals v d on dose of tet aca n (0 5 5t top cally on the cornea of each eye ju t befo e ca nul t on The cannul t on a performed b nt oduc ng a fine needle (outer d ameter 0 47 mm) w th an open g n its id into th anterior chambe The needle wa connected with sal ne filled polyethylene tubing (P E 20) to an el ctr cal pres u e transducer (Swema SP 23 D) and furthe on to a e ervo r (F g 2) The reservoir as adjusted to cause a pressure of 5 mm Hg in the sy t m to p event aqueous loss from the ye nto th can nula The needle and the manomet r re clo ed off f om th r servo r as soon a th needl was p ope ly positioned in the eye The electr cal outputs of the transducer ere regıste ed w th a reco d r (Metrohm E 478)

After cannulation the IOP a allowed to tabilize for at least 20 m 1 Dur n th s pe 1d th IOP wh ch had been increa ed by int oduc ng the n edle nto th anterior ch mber decreased to its original pr ssure level (P) A sal ne infu on wa then introdu ed nto th ye f om the reservo r at a known rat in o der to calculate th inflow r te a d outflo f c lit y (Fig 2) The speed of the nfu ion wa calculated by follow ng the mo em nt of a bubble n the poly th lene tub ng T

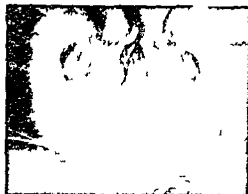


Fig 3



Fig 4

Fig 3 Experimental animal 5 days after the ster otaxic coagulation of the right oculomotor nerve *Note the maximal mydriasis in the right eye*

Fig 4 Microangiographic pre entation of the blood vessels in the anterior uvea 5 days after ciliarectomy Note the intact blood vessels (The authors want to thank Dr M Korman M D f r making this X ay microphotograph)

fusion rat (ΔF) was adjusted to about 2—3 μl/min With this infusion rate the original IOP (P) increased about 6 to 8 mm Hg which represented the new equilibrium pr sure (P_e) This equilibrium pr sure level was usually reached within 5—15 min after the onset of the infus on The reservoir was then closed off and the intraocular pressure (P) was obtained usually within 15 min If P_x diff red from P by more than 1 mm Hg the experiment was discarded The calculations were car i d t according to the formulae given by Sears and Barány (1960) C = ΔF/ΔP (ΔF = particular infusion rate ΔP = pr ssure change C = outflow facility) The values for aqueous inflow (F) were derived from the formula F = C (P − P) In this formula P is the episcleral pressure which was as umed t b c nstant and about 9 mm Hg (Kornbluth and Linnér 1955)

Aft r the measurements of IOP and inflow rate and outflow facility the animals were killed by an intra n ous air inject on Both eyes were removed in a cold room (4 C) and opened anteri r posteri rly into two identical hal es The ciliary body iris block was d ssected Of the 2 samples one wa cut nto tw id ntical halve and these were immediately imm ers d in liquid nitrogen and stored at −50 C unt l analyzed biochemically for ATPase The other half of the ciliary b d iris ring wa used f r histochemical stud es

Hi to he i al tud es Half of the ciliary body ir s block was cut into small radial pieces and these were u d t check hi tochemically the success of the denervations and to show the ATPases Adrenergic nerve f bres we e d monstrated also with a flu rescence technique n freeze-dri d specimens after paraformald hyde gass ng (Eranko 1967)

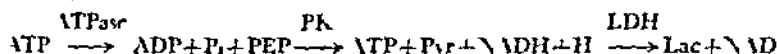
Some of the specimens were fixed for the dem n tratı n of neurotransmitter granules i KMnO containing solution (0 5 − 3 0) for 30 min The sections were cut after epon araldite embe ld ng and viewed w th an electr n microscope (Philips EM 300)

The tissue bl cks for hist chemical dem onst atı n f cholinesterases and ATPase were fixed in 2 5 buffered glutaraldehyde f r 2 hr t + 4 C and then rinsed in 0 2 M cacodylate buffered solution for about 20 hr b for sectıon ng and incul ating The ch linergic fib s were d m n trated with a m dif ed th och lin techn qu w th both light and lectron micr sc pes (Pa ama 19) ATPase activity w a dem onstrat d acc ding t a modified Wachste n Me el technique (Palk a and Uu italo 1970)

After ciliarectomy the blood ves els in the eye of som rabbits were studied by microang ography 2 and 5 days after the operation The right c r tid artery was prepare l and 10 cr 40 B s lphate in saline was injected int the artery at a pres ure of 110 mm Hg f r 30 min The eye then

embedded in paraffin and 100 μm sections were radiographed with a micro X ray tube (Kormano 1965).

ATP se ass y Frozen and sometimes fresh tissues were homogenized at 0 C in glass grinders with 10 volumes of 0 3 M mannitol for 1 5 min (Riley 1964) After homogenization activity was measured in one to two hr The experimental and control ciliary body iris were always homogenized and the activities measured at the same time The activity measurements were based on the following reaction scheme



$$ATP \xrightarrow{ATPase} ADP+P_i+PEP \xrightarrow{PK} ATP+Pyr+NADH+H \xrightarrow{LDH} Lac+NAD$$

The reaction was started by adding about 5 μl of homogenate equivalent to ca 60 μg of ciliary body iris protein to one ml of reaction mixture at 2? C The decrease in NADH fluorescence was observed in an Aminco-Bowman spectrophotofluorometer (excitation wave length 340 nm and fluorescence wave length 450 nm) equipped with a special adapter to take round 10 x 75 mm pyrex tubes (selected) The tubes were put into the fluorometer one after another for about five sec and the instantaneous peak of the fluorescence intensity of each tube was graphed on the recorder paper The velocity of the reaction in 10 to 15 tubes was observed for 10 to 15 min The reaction velocity became linear within one min of the addition of the homogenate and stayed linear for at least 15 min Appropriate blanks without ATP or homogenate or either were run simultaneously The enzyme activity was expressed as μmol/mg prot/min The protein was measured by the method of Lowry *et al* (1951)

Kinetic properties of ciliary body iris ATPase The reagent for preliminary ATPase study contained 50 mM Tris HCl buffer pH 8 1 3 mM amytal 2 5 mM MgCl 0 5 mM PEP ? 0 mM ATP (Na free) 0 05 mM NADH and 0 0? BSA (bovine serum albumin) When NaK ATPase activity was measured 10 mM Na and 30 mM K were used in the reagent

To obtain a small lag time and linearity of ATPase the auxiliary enzyme activities PK and LDH must be respectively 10 and 100 times higher than ATPase (Lowry *et al* 1961) For that purpose the PK and LDH activities were measured fluorometrically in one ml of reagent after diluting the enzymes with 50 mM Tris-HCl buffer pH 8 1 and 0 0 BSA Therefore 13 μg of LDH and ? μg of PK were normally used in ATPase assay From time to time the amount of auxiliary enzymes was checked by adding 2 to 3 times more of the enzymes to the reagent to see if the reaction velocity had decreased

Since commercial ATP preparations have some ADP as contaminant the addition of auxiliary enzymes always decreased the fluorescence by about 40 To measure NaK ATPase activity ATP Na was freed from Na using Dowex 50 H form After purification the pH of ATP H solution (about 70 mM) was standardized spectrophotometrically using hexokinase and glucose-6 phosphate dehydrogenase

Re ts The reagents used were from the following sources ATP (disodium salt) phosphoenol pyruvate (tri Cyclohexyl ammonium salt) pyruvate kinase hexokinase and glucose 6-phosphate dehydrogenase from C F Boehringer and Soehne G m b H Mannheim W Germany oxidized β-hyd on cotinamide adenine dinucleotide (B-NADH disodium salt) and bovine serum albumin from Sigma Chemical Co St Louis Mo U S A lactate dehydrogenase (BHLDH from Worthington Biochemical Co U S A All other chemicals used were obtained from commercial sources and were of analytical grade

Definition of ATPa items Because NaK ATPase activity cannot be determined directly in a crude preparation such as that used in this study the net NaK ATPase was estimated as the difference between total ATPase (measured in media with 10 mM Na 30 mM K and ? mM Mg and Mg ATPase (measured in Na and K free media)

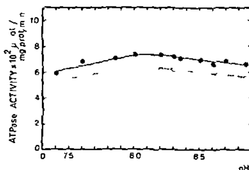
Statistical evaluation Experiments involving group 1 (sympathectomy group and group ? (ciliar ectomy group) were statistically analysed with a matched pair t test (Richterich 1968) This method was used to test whether the differences between the left and right eyes of the same rabbit differed significantly from zero Group 3 (parasympathectomy group was analysed by the t test between two means (De Jonge 1963) In this method a group of experimental (right) eyes was compared with the normal (left) eyes of other rabbits

Results

ATPase Assay

Optimal conditions In the reagent described above the conditions were such as to give activities that were linearly proportional to the time of incubation and the amount of enzyme

The effect of the following 50 mM buffer with different pH values was



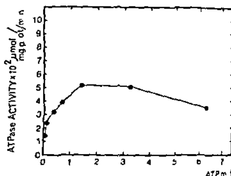


Fig 5 Fig 6

Fig 5 Effect of pH on the ATPase activity of ciliary body iris The homogenates were incubated at 22 C in 1 ml of reagent containing 3 mM amytal 1 9 mM $MgCl_2$ 0 5 mM PEP 1 6 mM ATP 0 05 mM NADH and 0 02 BSA The pH of 50 mM Tris HCl buffer was varied from 7 4 to 8 8 Closed circles with 10 mM Na and 30 mM K (total ATPase) open circles correspond to incubation in the absence of Na and K (Mg ATPase)

Fig 6 Effect of varying ATP concentrations on total ATPase activity at pH 8 1 Cond tions as in Fig 5 except that ATP was varied from 0 1 to 6 3 mM

Imidazol HCl pH 6 5 and 7 5 phosphate buffer pH 7 0 and 7 5 Tris HCl buffer pH 7 4, 7 6 7 8 8 1 8 3 8 5 8 8 and glycine buffer pH 8 5, 9 0 and 9 5 The optimal pH was 8 1 for both Mg ATPase and NaK ATPase (Fig 5) Tris HCl was the most suitable buffer

The optimal Mg ATP ratio for Mg ATPase and NaK ATPase was determined by varying both ATP concentration from 0 1 to 6 3 and Mg^{++} concentration from 0 1 to 9 6 mM ATP and Mg^{++} were used in final concentrations of 1 6 mM and 1 9 mM respectively (Figs 6 and 7) The optimal Mg/ATP ratio was ca 1 for both Mg ATPase and NaK ATPase (Fig 8)

The effect of varying the concentrations of Na^+ and K^+ on the ATPase activity are shown in Figs 9 and 10 A specific fraction of the total activity was dependent upon the presence of both Na^+ and K^+ This fraction was also inhibited by 3×10^{-4} M

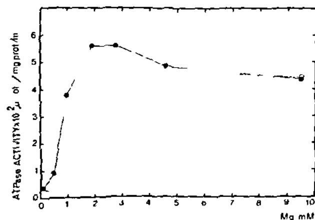


Fig 7 Effect of varying Mg c n centrations on ATPase activity at pH 8 1 Cond tions and symbols as in Fig 5 except that Mg was varied from 0 1 to 9 6 mM

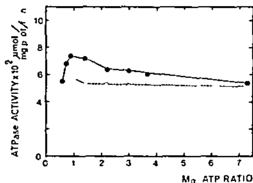


Fig 8 Effect of Mg/ATP ratio on ATPase activity at pH 8 1 Conditions and symbols as in Fig 5 except that Mg/ATP ratio was varied from 0 6 to 7 8

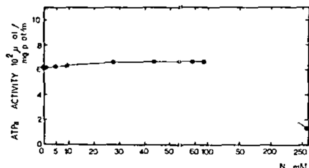


Fig 9 Effect of Na concentration on ATPase activity at pH 8 1 Conditions as in Fig 5 except that Na was varied from 0 1 to 250 mM K concentration was constant (30 mM)

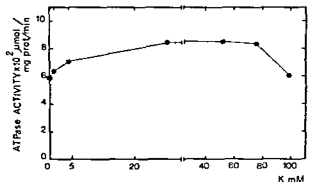


Fig 10 Effect of K concentration on ATPase activity at pH 8 1 Conditions as in Fig 5 except that K was varied from 0 1 to 100 mM Na concentration was constant (10 mM)

ouabain (Fig 11) Inhibition was not enhanced when ouabain was present but not Na^+ or K^+

Amytal was originally included in the reagent media to inhibit the NADH oxidase activity of tissue homogenate Later it was noticed that 3 mM amytal inhibited about 40 % of the NaK ATPase activity and about 5 % of the Mg ATPase activity

ATPase is known to be sensitive to temperature (Willis and Ma Li 1969) We made some incubations at 37 C so as to be able to correlate the results at 22 C with

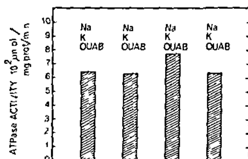


Fig 11

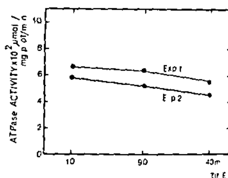


Fig 12

Fig 11 Effect of 3 × 10 M ouabain and different cations (Na K and Mg) on ATPase activities Conditions as in Fig 5 The incubation was carried out at pH 8 1

Fig 12 Effect of storage of the homogenate at 0 C on total ATPase activity Conditions as in Fig 5 The incubation was carried out at pH 8 1

those at 37 C When the assay temperature was increased from 22 to 37 C ATPase activity almost doubled

During preliminary studies it was noticed that storing ciliary body iris blocks at −50 C for periods of up to two weeks did not affect the ATPase activity After tissue homogenization storage at 0 C somewhat decreased ATPase activity (Fig 12)

Histochemical findings after various denervations The success of the sympathectomy was checked by demonstrating the fluorescent fibres in the ciliary body iris block using fluorescence microscopy Fluorescent fibres disappeared from the ciliary body almost totally 24 hr after the operation Two days afterwards all the fluorescent fibres had been destroyed whereas in the contralateral (left) side the fluorescent nerves were intact

Ciliarectomy did not cause such a total disappearance of the histochemically demonstrable nerve fibres In about 60 % of the operated animals both cholinergic and adrenergic fibres clearly decreased The cholinergic denervation seemed to be more complete than the adrenergic Cholinergic and adrenergic fibres had completely disappeared in only two of the 30 rabbits operated on

After parasympathectomy by the stereotaxic technique special attention was paid to the reaction of the pupil Only animals in which a large mydriasis was recorded and whose pupils did not react to light were accepted Histochemically enzyme activity increased on the proximal side of the coagulation area in the right optic nerve In the ciliary body iris block no histochemically demonstrable cholinesterase changes were observed The number of fluorescent fibres was also unchanged In many successfully operated animals the left oculomotor nerve had also been partly damaged The left eye was therefore not accepted as a control eye in this group

In the microangiographic studies made after ciliarectomy the long posterior ciliary arteries seemed to be well filled as did the arterial circle in the iris area Microscopically the blood vessels in the ciliary body area appeared well filled and no circulatory disturbance could be observed (Fig 4)

TABLE IA Effect of sympathectomy (group 1) on intraocular pres ure (IOP) outfl facil t (C) and infl w (F)

Time after Denervation in Days	IOP mm Hg Control Eye	IOP mm Hg Experimental Eye	IOP Mean Difference	C µl min/mm Hg Control Eye	C Experimental Eye	C Mean Difference	F µl/min Control Eye	F Experimental Eye	F Mean Difference
1	(4) 13 8±0 9	14 3±0 8	−0 5±0 3	(4) 0 38±0 07	0 27±0 02	0 11±0 05	(4) 1 75±0 36	1 41±0 21	0 34±0 25
2	(7) 12 9±0 6	12 8±0 5	0 1±0 7	(7) 0 31±0 04	0 24±0 03	0 08±0 04	(7) 1 14±0 17	0 82±0 07	0 33±0 13
5	(5) 15 3±0 5	15 5±0 5	−0 2±0 2	(5) 0 31±0 07	0 42±0 08	−0 13±0 06	(5) 1 90±0 58	2 9±0 60	−0 89±0 43
30	(2) 18 2±3 0	16 1±1 2	2 1±0 9	(2) 0 15±0 02	0 32±0 04	−0 17±0 06	(2) 1 29±0 27	2 06±0 11	−0 7 ±0 16

Number of experim nts in parentheses Statistical analyses revealed no significant d fferences bet ween paired eyes Standard errors are those of the mean

The NaK ATPase activity demonstrated histochemically at the membranes of the epithelial cells of the ciliary processes had not detectably decreased after sympa thectomy However ciliarectomy clearly decreased enzyme activity After the stereo taxic parasympathetic denervation also NaK ATPase activity clearly decrea ed

Intraocular fluid dynamics and ATPases Intraocular fluid dynamics and biochemical analyses of ATPase showed marked individual variation In selecting the animals for the different groups we tried to use animals of the same age and weight for similar types of experiment We found that the most reliable control for the experimental eye was its contralateral eye Thus the results obtained from one group cannot easily be compa

TABLE IB Effect of sympathectomy (group 1) on ATPase acti ty in the ciliary body

Time after Denervation in Days	Total ATPase 10 µmol/mg prot/min Control Eye	Total ATPase Experimental Eye	Total ATPase Mean Difference	Mg ATPase 10 µmol/mg prot/min Control Eye	Mg ATPase Experimental Eye	Mg ATPase Mean Difference	NaK ATPase 10 µmol/mg prot min Control Eye	NaK ATPase Experimental Eye	NaK ATPase Mean Diff
1	(3) 4 92±1 25	5 03±0 25	−0 12±1 45	(3) 3 77±0 77	3 56±0 10	0 21±0 86	(3) 1 14±0 51	1 47±0 20	−0 33±0 58
2	(7) 6 00±0 38	5 14±0 30	0 86±0 32	(7) 4 72±0 31	4 02±0 26	0 71±0 30	(7) 1 28±0 16	1 12±0 20	0 15±0 16
5	(6) 5 00±0 54	5 09±0 19	−0 09±0 41	(6) 3 70±0 33	3 86±0 15	−0 16±0 8	(6) 1 30±0 25	1 23±0 13	0 07±0 15
30	(4) 4 50±0 54	4 72±0 34	−0 2±0 31	(4 3 63±0 53	3 58±0 33	0 12±0 37	(4) 0 87±0 07	1 14±0 13	−0 27±0 19

Number of experiments n parentheses Stat t cal analyses re ealed no significant differences bet ween paired eyes Standard errors are tho of the mean

1962 a, Willis and Ma Li 1969) We used neither of these techniques however but rather a fluorometric method This was mainly due to the many advantages of the fluorometric technique The fluorometric assay for ATPase is not only sensitive and simple to use but is also kinetic and linear for long periods of the reaction (even 1 hr)

In this fluorometric technique the ATPase activity was measured by following the decrease in NADH fluorescence Because the NADH oxidase activity in tissue homogenates also decrease NADH fluorescence, 3 mM amytal was used to inhibit this non specific enzyme activity Amytal also inhibited NaK ATPase activity by about 40 % and Mg ATPase activity by about 5 % This inhibitory effects of amytal on ATPase activities were however, so constant that the actual activities of the enzymes could be calculated This amytal induced inhibition of the ATPase activity of the ciliary body iris corresponds well to the findings of Jarnefelt (1962 b) and Schwartz and Laseter (1964) who studied the enzyme activity in other organs

ATPase activity in the ciliary body Since the early works of Bonting *et al* (1961) it has been assumed that NaK ATPase plays a part in the active ion transport mechanism in the ciliary epithelium This ciliary body iris NaK ATPase activity in fact seems to be very similar to the transport enzyme in other organs The ciliary body iris NaK ATPase enzyme requires Mg^{++} Na^{+} and K^{+} Because the enzyme was also sensitive to ouabain it seemed to have all the characteristics of the pump enzyme (Skou 1957)

The NaK ATPase activity in the ciliary body iris of the rabbit had similar activity to the enzyme in rabbit pancreas (Ridderstap and Bonting 1969) On the other hand the enzyme in rabbit kidney rabbit brain and cat choroid plexus has been reported to be more active than ciliary ATPase (Bonting *et al* 1964 Vates *et al* 1964 Bonting and Becker 1964) The low activity of ciliary iris NaK ATPase compared for instance with that in rabbit brain may possibly be explained by the totally different structural characteristics of these two organs In the ciliary body iris the epithelial cells responsible for electrolyte and water secretion of aqueous humour are in a minority compared with the stroma The percentage of the total ATPase activity that is NaK ATPase varies in different organs In the ciliary body iris it is about 25 % whereas in the brain it is about 40 % (Bonting *et al* 1962)

Intraocular fluid dynamics and ATPase activity Dissection of the superior cervical ganglion (*sympathectomy*) did not significantly change the IOP when the operated eye was compared with the contralateral eye However the IOP was relatively low in both eyes of rabbits in this group 1 and 2 days after the operation In this group the outflow facility in the experimental eye was slightly decreased two days after the operation However this change was later completely reversed since the outflow facility was increased 5 days after the operation These findings are in good agreement with those of Linner and Prijot (1955) On the other hand Langham and Taylor (1959 1960 a, b) and Sears and Barany (1960) reported a distinct increase in outflow facility 24 hr after sympathectomy We also found a change in the same direction though at

a later stage (5 days). Sympathectomy did not alter the inflow of aqueous humour and the ATPase activities in the ciliary body iris also remained unchanged.

Dissection of the ciliary nerves (*ciliarectomy*) markedly decreased the IOP 2 and 5 days after the operation. This decreased IOP was followed by a marked decrease in inflow, e.g. 5 days after the operation the inflow was 0.13 μl/min versus 1.96 μl/min. However, this change was not statistically significant, apparently due to the small number of animals. Furthermore, the NaK ATPase activity had also decreased and this change was significant. From these results we conclude that ciliarectomy decreased IOP possibly due to inhibition of NaK ATPase activity. This in turn caused a decreased inflow. Changes in the same direction were also produced by parasympathectomy alone. In the parasympathectomy group IOP and enzyme activity had decreased whereas the inflow had not changed much.

From these results it appears that the changed IOP, NaK ATPase activity and possible inflow are in closer relationship with the parasympathetic than with the sympathetic nerves. This was stated earlier by Uusitalo and Palkama (1971).

The decreased IOP following parasympathectomy may also reflect changes in the eye other than the decreased inflow, for instance changes in the episcleral pressure (P_v) and the relaxation effect of the extraocular muscles of the eye. If parasympathectomy is assumed to cause such a relaxation of the extraocular muscles that the IOP is decreased, the compensating mechanism of the eye would return the IOP to its original level within a few minutes (Barany 1947, Perkins 1957, Sears 1960). However, the change in IOP after parasympathectomy was observed even 5 days after the operation. On the other hand, the possibility that parasympathectomy decreased episcleral venous pressure cannot be excluded. Because the active transport mechanism in the inflow system has changed markedly, it easily explains the decreased IOP. The episcleral pressure possibly plays a minor role in this regulating system.

We have assumed that parasympathetic denervation decreases NaK ATPase activity in the ciliary body iris. How the "pump" enzyme activity is dependent on the parasympathetic nerves is difficult to answer on the basis of our present results. However, the effect of denervation on other tissues and their biochemical compositions has been studied (Gutman 1962). Axelsson and Thesleff (1959) and Thesleff (1960) have studied the spread of acetylcholine (ACh) sensitivity in denervated muscle. Guth (1968, 1970) has shown that the level of enzyme activity in muscle is dependent on its specific innervation. He stated that the nerve regulates the qualitative type of actomyosin ATPase synthesized by the muscle fibres. We cannot conclude whether the synthesis of ciliary epithelium ATPase activity is regulated by the nerves innervating these cells, but this is a tempting theory and would explain our results.

Removal of the ciliary ganglion or intracranial section of the oculomotor nerve results in a marked ACh sensitization of the iris sphincter (Keil and Root 1941, Cannon and Rosenblueth 1949). This sensitization reaches a maximum about 5 days after denervation and the iris seems to behave like muscle after its denerva

tion We found that the NaK ATPase activity in the ciliary body iris decreased after ciliarectomy or coagulation of the oculomotor nerve The lowest values for NaK ATPase activity were 2 to 5 days after the operation The changes in NaK ATPase activity seem to occur at the same time as the supersensitivity to ACh is recorded in the eye

Evidently NaK ATPase activity in the ciliary body iris is responsible for the transport of ions into the posterior chamber of the eye The NaK ATPase activity is located in the membranes of the non pigmented epithelial cells of the ciliary body (Palkama and Uusitalo 1970) This enzyme activity is significantly decreased after parasympathetic denervation This change occurs simultaneously with a decreased IOP and inflow of aqueous humour It seems likely that these three phenomena are inter dependent

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Abbreviations (ATPase=adenosine triphosphatase PEP=phosphoenolpyruvate PK=pyruvate kinase Pyr=pyruvate NADH and NAD=reduced and oxidized dihydronicotinamide adenine dinucleotide LDH=lactic dehydrogenase Lac=lactate)

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In Vitro Work Load and Rat Heart Metabolism

III Effect on ribosomal aggregation

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Abstract

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In two preceding papers increased pressure load (afterload) was found to accelerate whole heart protein synthesis and amino acid transport in the perfused working rat heart (Hjalmarson and Isaksson 1972 Ahrén *et al* 1972) To further analyse this effect sucrose gradient analysis of the postmitochondrial supernatant was performed to evaluate reactions involved in the ribosome cycle When hearts were perfused *in vitro* under control conditions with buffer containing normal plasma levels of amino acids and with glucose as substrate levels of polysomes decreased and levels of ribosomal subunits increased These findings together with a decreased rate of protein synthesis indicated that a block in initiation of peptide chains had developed during perfusion Perfusion of hearts with increased afterload increased the levels of polysomes and decreased the levels of ribosomal subunits and accelerated protein synthesis The effects were obtained both when glucose and palmitate were used as substrate Insulin further increased the levels of polysomes in pressure overloaded hearts and the ribosome profiles attained by overload in presence of insulin were identical to those obtained from unperfused hearts Increased levels of polysomes and decreased levels of ribosomal subunits together with an increase in protein synthesis suggested that pressure overload stimulated reactions involved in the initiation of polypeptide chains It is concluded that increased pressure load increases levels of initiation factors or changes the activity of enzymes regulating initiation processes in the ribosome cycle

An increase in the afterload of the isolated working rat heart accelerates the protein synthesis and the membrane transport of amino acids (Hjalmarson and Isaksson 1972 Ahrén *et al* 1972) The effect on membrane transport of the non utilizable amino acids a aminoisobutyric acid (AIB) and cycloleucine was obtained when protein synthesis was blocked by cycloheximide This might indicate that an increase in membrane transport of amino acids in overloaded hearts is not directly dependent upon a continuous synthesis of protein and that the increased uptake of amino acids is not secondary to the work load effect on protein synthesis Changes in the transport rate of amino acids into the heart cells could be an important mechanism through which increased physiological demand results in cardiac hypertrophy However other postulated rate limiting steps in the regulation of heart

protein synthesis might as well be involved as intracellular amino acid activation peptide chain elongation and transcription proces es

It seems now generally accepted that the rate of protein synthesis in mammalian cells can be regulated at the ribosomal level (*e g* Earl and Korner 1966) For the heart muscle cells it has recently been shown that *in vitro* incorporation of phenyl alanine was reduced after hypophysectomy and that injections of growth hormone or thyroxine to the hypophy ectomized rats could restore this defe t Ribo omal aggregation and disaggregation paralleled these changes in the rate of protein syn thesis (Hjalmarson *et al* 1970) Perfusion of rat hearts *in vitro* in pre ence of in sulin palmitate or increased levels of all normal amino acids increased the rate of heart protein synthesis (Rannels *et al* 1970 Morgan *et al* 1971 a b) and con currently the ribosomal aggregation The afore mentioned tudies and a number of other investigations have shown that there is a correlation between the rate of pro tein synthesis as well in mammalian cells as in bacteria and the number of poly somes When the number of polyribo omes is increa ed the pro ein synthesis is ac celerated (Hogan and Korner 1968 a Pronczuk *et al* 1968)

Evidence from studies in bacteria and eukaryotic cells suggests that the ribo omal system consists of three components polyribosomes ribosomes and ribosomal sub units (Mangiarotti and Schlessinger 1967 Kaempfer *et al* 1968 Hogan and Korner 1968 a b Kabat and Rich 1969) A continuous cyclic association and dissociation occurs between ribosomal subunits ribosomes and polyribo omes both in bacteria and in eukaryotic cells (Girard *et al* 1965 Kabat and Rich 1969) In mammalian cells the monomer of the ribosome is of the 80 S type which dissociates into a larger 60 S subunit and a smaller 40 S subunit The aggregation of the ribo omal subunits to ribosomes at the initiator codon the movement of the ribosomes along the messenger RNA (mRNA) during peptide chain elongation and the release of completed chains at the terminator codon constitute the main steps of the process in protein synthesis that has been designated the ribo omal cycle The rate between initiation of ribo- somal aggregation in relation to the rate of elongation termination of the peptide chains determines the levels of polysomes and ribosomal subunits

Low levels of subunits result when initiation is rapid relative to elongation and *vice versa* In the present study the effects of increased *in vitro* work load on the ribo omal cycle activity of the heart muscle was investigated The cycle was con sidered to consist of two groups of reactions those involved in (i) peptide chain initiation and (ii) peptide chain elongation If the rate of protein synthesis and the relation between ribosomal subunits and polysomes are known it can be deduced whether changes in the *in vitro* work load of the heart affects the activity in the ribosomal cycle

Methods

Experimental procedure

Hearts were removed from fed female rats of the Sprague Dawley strain (200—250 g) anesthetized with Nembutal (60 mg/kg b w) The he rts were rap dly excised and transferred to ice-chilled sal ne and the aorta and left at ium w re cannulat d After a retrograde wash

out period of 5 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) the hearts were perfused in the forward direction at 37 C as described previously (Neely *et al* 1967 Hjalmarson and Isaksson 1972) The perfusion medium was Krebs Henseleit bicarbonate buffer containing the disodium salt of EDTA (0 5 mM) with all amino acids at normal rat plasma levels (Morgan *et al* 1971 a) and with glucose (14 mM) or palmitate (1 5 mM) as substrate When palmitate was used a fatty acid albumin complex was prepared as described previously (Hjalmarson and Isaksson 1972) The recirculating volume (40 ml) was continuously gassed with 95 % O_2 and 5 % CO equilibrated with water at 37 C The control hearts were perfused at a left atrial filling pressure of 10 cm H_2O and were forced to pump the fluid against a hydrostatic pressure head of 70 cm H_2O Pressure overloaded hearts (increased afterload) were perfused at the same left atrial filling pressure but with the aortic tube totally constricted By this increase in afterload the peak systolic pressure increased from about 95 mm Hg in the control hearts to about 160 mm Hg in the overloaded hearts There was a concomitant increase in the aortic diastolic pressure from approximately 45 to 120 mm Hg and an increase in the coronary flow from 75 to 200 ml/g d w × min The heart rate was approximately 240 beats/min both in the control and in the overloaded hearts Aortic pressures and heart rate were recorded by a transducer attached to a manifold of the aortic tube and the pressure tracings were registered by a Sanborn recorder model 16 1300 S Coronary flow was measured by collecting the fluid dropping from the heart

Phenylalanine ^3H(C) was obtained from the New England Nuclear Corp Boston USA Labelled and nonlabelled phenylalanine was added to the medium to make a final molarity of 0 08 mM and a specific activity of 1 25 μC/μmole The incorporation of phenylalanine ^3H into heart protein was determined as described previously (Hjalmarson and Isaksson 1972) and is expressed as DPM/mg protein × 10^{-3} A 10 times recrystallized porcine insulin was used (Novo lot No 823267)

Sucrose gradient analysis of heart ribosomes

Sucrose gradient analysis of the postmitochondrial supernatant was performed as described by Morgan *et al* (1971 b) Hearts were cut in a beaker containing ice-chilled 0 25 M KCl and 2 mM $MgCl_2$ in 10 mM Tris buffer at pH 7 40 The following procedures were carried out in a cold room at 2 C The hearts were opened rinsed and the large vessels and valves were trimmed away The muscles were blotted on filter paper and minced with scissors Three hearts were pooled for each homogenate to obtain sufficient tissue The tissue was homogenized in three volumes of 10 mM Tris buffer containing 2 mM $MgCl_2$ and 0 25 M KCl in a Dounce homogenizer (40 ml) using a Teflon ball mounted on a plastic rod Six strokes with a loose fitting ball and 6 strokes with a tight fitting ball were required The homogenate was spun at 2 C for 10 min at 8000 × g in a refrigerated centrifuge Triton X 100 was added to the supernatant to give a final concentration of 0 3 % Of this supernatant 0 75 ml was layered onto each of three exponential sucrose gradients formed in tubes of a SW 41 rotor (Spinco) using 15 to ? M sucrose dissolved in the homogenization buffer Following centrifugation at 40 000 rpm for 3 1/4 hr the gradients were monitored by pumping the contents of the tube through a flow cell of a Gilford recording spectrophotometer at 260 mμ In some experiments the effluent from the flow cell was collected in fractions for subsequent analysis of the RNA content

Analysis of total RNA content

Total RNA content was determined by alkaline hydrolysis as described by Fleck and Munro (1962 When gradient fractions were analysed 0 5 ml of 0 5 bovine serum albumin was added to each fraction and was carried through the whole analysis procedure The same amount of albumin served as a blank The proteins and nucleic acids were precipitated in 10 trichloroacetic acid (TCA) and the precipitate was washed twice in 3 ml of 10 % TCA The final precipitate was incubated with 3 ml of 0 3 N KOH in a water bath at 37 C for 60 min to hydrolyse the RNA After the incubation 1 ml of 4 N perchloric acid (PCA) was added to precipitate the DNA and the protein of the digest

The absorbance of the supernatant was determined at 260 and 280 mμ and the RNA content was calculated supposing $E^{1\%}_{1\,cm}$ to be 213 (extinction coefficient for a 1 % RNA solution at 260 mμ in a 1 cm cuvette) as in purified rat liver RNA (Fleck and Munro 1962) The total RNA content of whole heart homogenate varied slightly from experiment to experiment. An average value was found to be 3 05 ± 0 31 mg/g heart homogenate (3 expts) The RNA content is expressed as mg RNA/3 mg RNA to be able to compare different experiments and to statistically analyze the RNA content of the polysomes the two subunits and the supernatant In similar experiments Morgan *et al* (1971 b) also found the RNA content to be about

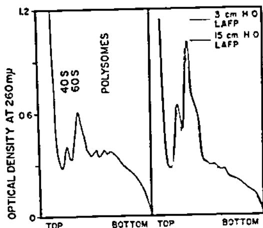


Fig 1 Sucrose gradient analysis of heart ribosomes The left part of the figure demonstrates pattern of ribosomal aggregation obtained from unperfused hearts Positions of the polysomes and the large (60 S) and small (40 S) subunits are indicated on the figure The right part of the figure shows pattern of ribosomal aggregation in hearts perfused with different preloads Hearts were perfused for 60 min with Krebs-Henseleit bicarbonate buffer containing glucose (15 mM) and all amino acids at normal plasma levels Hearts were homogenized and sucrose gradient analysis carried out as described in Methods The experiments were performed several times

3 mg/g homogenate The E ₂₆₀/E ₂₈₀ ratio of corresponding fractions did not differ between control and overloaded hearts

Statistics

Analysis of variance (with one criterion of classification) followed by the Student Newman Keuls multiple range test was used (Woolf 1968) A p-value of 0 05 or less was considered significant in this study

Results

The left part of Fig 1 shows the ribosome profiles of unperfused hearts In these hearts the levels of the large and small subunits are low relative to the level of polysomes During perfusion with glucose there is a progressive decrease in the rate of phenylalanine incorporation in the perfused rat heart (Morgan *et al* 1971 a Hjalmarson and Isaksson 1972) By adding palmitate or amino acids 5 times the normal plasma level to the perfusate or by increasing the afterload the protein synthesis was accelerated On the other hand an increase in the preload did not stimulate the protein synthesis (Hjalmarson and Isaksson 1972) As can be seen from the right part of Fig 1 there was an increase in the levels of ribosomal subunits and a decrease in the levels of polysomes in hearts perfused for 60 min with glucose compared to unperfused hearts The disaggregation of polysomes was more pronounced after a perfusion period of 120 min (not shown in the fig) A similar disaggregation of polysomes with time has been reported to occur in the retrogradely perfused heart (Morgan *et al* 1971 b) The progressive disaggregation of polysomes and the concomitant decrease in protein synthesis under similar conditions to those in Fig 1 suggests that a block in peptide chain initiation was involved in the inhibition of protein synthesis as has been proposed by Morgan *et al* (1971 b) It is further seen from the right part of Fig 1 that an increase in the left atrial filling pressure from 3 to 15 cm H O did not change the pattern of ribosomal aggregation

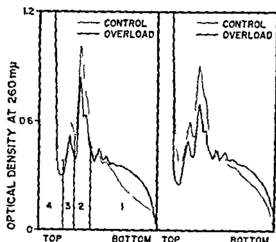


Fig. 2. Effects of increased pressure work load on ribosomal aggregation in hearts perfused with glucose (left panel) or palmitate (right panel) as substrate. Hearts were perfused for 60 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) or palmitate (1.5 mM) and all amino acids at normal plasma levels. The portions of gradients collected for RNA analysis are indicated as (1) polysomes and monomeric ribosomes, (2) large ribosomal subunit, (3) small ribosomal subunit and (4) supernatant. The experiment in the left panel and in the right panel was repeated 6 and 2 times, respectively.

The left part of Fig. 2 demonstrates that the number of subunits decreased and that the levels of polysomes increased in the overloaded hearts after 60 min of perfusion with glucose as substrate. As was shown previously (Hjalmarson and Isaksson 1972) and can be seen from Table I, this elevated pressure work load stimulated the incorporation of phenylalanine-^{3}H into the proteins of the heart. The stimulatory effect of pressure overload on the rate of protein synthesis was obtained both with glucose and palmitate as substrate and when the normal level of amino acids in the perfusate was increased 5 times (Hjalmarson and Isaksson 1972). Inclusion of palmitate in the perfusate and increased levels of amino acids have been shown to stimulate the protein synthesis in the heart (Morgan *et al.* 1971; Rannels *et al.* 1970; Hjalmarson and Isaksson 1972). The increase in the polysome to ribosomal subunit ratio (Fig. 2) together with high incorporation rates of phenylalanine-^{3}H suggests that increased pressure work facilitated peptide chain initiation.

Spectrophotometric analysis of the sucrose gradient profiles could be misleading since other material with absorption at 260 mμ could sediment along with the ribosomes and variations in the base line absorbance could occur. To overcome some of these uncertainties direct measurements of the total RNA content in the different gradient fractions of the sucrose gradient profiles were performed. Fractions were collected as indicated in the left panel of Fig. 2. Frequently a distinct notch was seen in the optical density pattern of fraction no. 2 which might indicate the existence of a monomeric ribosome in the large subunit fraction. However, this did not influence the relation of polysomes to ribosomal subunits between control and overloaded hearts since equal volumes were collected in corresponding fractions from the gradients. Table II shows that the RNA c[illegible]nt increased in the polysome fraction and [illegible] in th[illegible] subunit fraction in hearts exposed to pressure overload for 6[illegible] tro[illegible]ficant change in the total RNA content of th[illegible] small subu[illegible] or [illegible]atant fraction were seen between control and over-load.

TABLE I Effect of increased pressure work load on the incorporation of phenylalanine H into heart protein of isolated working rat heart in presence and absence of insulin

Experimental cond tion	Insul n 10 mU/ml	Phenylalanine ³H incorporation rate DPM/mg protein × 10 ³	
Control	–	252 ± 8	p<0 05
Control	+	319 ± 8ᵇ	
Pressure overload	–	374 ± 11ᶜ	p<0 05
Pressure overload	+	363 ± 7ᵈ	

Hearts were perfused anterogradely for 90 min with Krebs Henseleit b carbonate buffer containing glucose (14 mM) and all am no acids at normal plasma levels in abs nce and presence of insulin Values are mean ± S E Six hearts in each group a vs c p<0 05 b vs d p<0 05

To investigate if increased pressure load on the heart stimulated the protein synthesis to a maximal rate control and pressure overloaded hearts were perfused with and without insulin as shown by Table I It can be seen from this table that insulin further stimulated the incorporation of phenylalanine in the overloaded hearts to a small extent and that insulin also markedly stimulated the phenylalanine incorporation in the control hearts

When sucrose gradient analysis was performed under the same experimental conditions with the exception that perfusion time now was 60 compared to 90 min in the incorporation experiment insulin gave a pronounced increase in the levels of polysomes and a decrease in the levels of ribosomal subunits of the control hearts as shown by the left part of Fig 3 The effect of insulin on the pattern of ribosomal aggregation in overloaded hearts is difficult to evaluate but the right part of Fig 3 shows that a small increase in the levels of polysomes in the presences of insulin might have occurred The ribosome profiles attained by overload in presence of insulin were nearly identical to those obtained from unperfused hearts as shown by Fig 1 which indicates that the protein synthesi can be fully maintained if the hearts are perfused under optimal conditions

TABLE II Effects of increased pressure work load on RNA content of sucrose gradient fract ons

Sucrose gradient peaks	Control				Pressure overload
	mg RNA/3 mg RNA in he rt homogenate				
Fraction no 1	0 209 ± 0 076	—	p<0 05	—	0 311 ± 0 072
Fraction no 2	0 377 ± 0 021	—	p 0 05	—	0 282 ± 0 019
Fract on no 3	0 187 ± 0 017	—	N S	—	0 131 ± 0 016
Fraction no 4	0 464 ± 0 023				0 486 ± 0 049

Hearts were perfused for 60 min with Kr bs Hensel t b carbonate buffer containing glucose (14 mM) and all am no acids at normal plasm a le ls F actions w ollected as ndic ted in F g 2 and tot l RNA c ntent was measured Collection f the supernatant f ction was continued until the optical density returned to the base lin E h hom g nate was ent f ged on three gradi nts and the fractions were analys d separately Thr hea ts were pooled for each homogen t nd th e per m nt was repeated 3 times Values a means ± S E

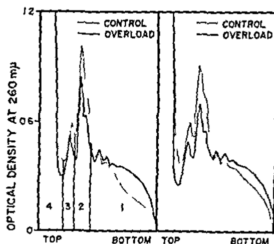


Fig 2 Effects of increased pressure work load on ribosomal aggregation in hearts perfused with glucose (left panel) or palmitate (right panel) as substrate Hearts were perfused for 60 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) or palmitate (1 5 mM) and all amino acids at normal plasma levels The portions of gradients collected for RNA analysis are indicated as (1) polysomes and monomeric ribosomes (2) large ribosomal subunit (3) small ribosomal subunit and (4) supernatant The experiment in the left panel and in the right panel was repeated 6 and ? times, respectively

The left part of Fig 2 demonstrates that the number of subunits decreased and that the levels of polysomes increased in the overloaded hearts after 60 min of perfusion with glucose as substrate As was shown previously (Hjalmarson and Isaksson 1972) and can be seen from Table I this elevated pressure work load stimulated the incorporation of phenylalanine ^{3}H into the proteins of the heart The stimulatory effect of pressure overload on the rate of protein synthesis was obtained both with gluco e and palmitate as substrate and when the normal level of amino acids in the perfusate was increased 5 times (Hjalmarson and Isaksson 1972) Inclusion of palmitate in the perfusate and increased levels of amino acids have been shown to stimulate the protein synthesis in the heart (Morgan *et al* 1971 Rannels *et al* 1970 Hjalmarson and Isaksson 1972) The increase in the polysome to ribosomal subunit ratio Fig 2) together with high incorporation rates of phenylalanine ^{3}H suggests that increa ed pressure work facilitated peptide chain initiation

Spectrophotometric analysis of the sucrose gradient profiles could be misleading since other material with absorption at 260 mμ could sediment along with the ribosomes and variations in the base line absorbance could occur To overcome some of the e uncertainties direct measurements of the total RNA content in the different *gradient fractions of the sucrose gradient profiles were performed* Fractions were collected as indicated in the left panel of Fig 2 Frequently a distinct notch was seen in the optical density pattern of fraction no 2 which might indicate the existence of a monomeric ribo ome in the large subunit fraction However this did not influence the relation of polysomes to ribosomal subunits between control and overloaded hearts since equal volumes were collected in corresponding fractions from the gradients Table II shows that the RNA content increased in the polysome fraction and decreased in the large subunit fraction in hearts exposed to pressure overload for 60 min *in vitro* No significant changes in the total RNA content of the small subunit fraction or of the supernatant fraction were seen between control and overloaded hearts (Table II)

tide chain elongation It has been reported earlier (Schreiber et al 1967) that isolated microsomes from acutely overloaded hearts had a better capacity to synthetize proteins in vitro compared to microsomes from control hearts This could be due to changes in ribosomal aggregation as described in this study

Retrograde perfusion of the isolated rat heart has been shown to be associated with an increase in the levels of ribosomal subunits and a decrease in the level of polysomes (Morgan et al 1971 b) A similar disaggregation of polysomes with time was evident from the present study using the working rat heart preparation (Fig 1) Under similar perfusion conditions there is a progressive decline in the protein synthesis (Morgan et al 1971 a Hjalmarson and Isaksson 1972) which indicates that a block in the peptide chain initiation develops during perfusion It could be argued that increased pressure work load prevented the disaggregation of polysomes rather than stimulated the reactions involved in the initiation of polypeptide chains However as pointed out before (Morgan et al 1971 b Hjalmarson and Isaksson 1972) perfusion conditions in vitro with glucose as substrate are not satisfactory for optimal rates of protein synthesis Factors known to be of importance for the protein synthesis in the heart as e g insulin (Morgan et al 1971 b) and fatty acids (Rannels et al 1970) are lacking during perfusion in vitro

In previous investigations (Hjalmarson and Isaksson 1972 Ahren et al 1972) it was found that increased preload did not accelerate the incorporation of phenylalanine ^3H into the heart proteins or increase the uptake of amino acid in the perfused heart In the present study it was shown that increased preload did not stimulate initiation of ribosomal aggregation On the contrary increased afterload accelerated the amino acid uptake and the incorporation of amino acids into tissue proteins and elevated the levels of ribosomal polysomes This parallelism between postulated rate limiting steps in the protein synthesis in the heart suggests that amino acid transport incorporation of amino acids into proteins and ribosomal aggregation are interrelated in a physiologically significant way

The presence of growth hormone thyroxine insulin perfusion with fatty acids as substrate instead of glucose and elevated levels of all normal amino acids stimulate the incorporation of amino acids into proteins of the isolated rat heart by favouring the initiation of ribosomal aggregation (Hjalmarson et al 1970 Rannels et al 1970 Morgan et al 1971 b) The finding in the present study that patterns of ribosomal aggregation in overloaded hearts and after perfusion of the hearts in presence of insulin or with palmitate as substrate were very similar raises the question whether insulin palmitate and increased pressure work stimulate protein synthesis through common mechanisms No definite conclusion can be drawn from the present study but the finding that insulin and palmitate further stimulated the protein synthesis in the pressure overloaded hearts might indicate qualitative differences between insulin palmitate and pressure overload concerning the stimulatory effect on protein synthesis

In the present study a significant effect of pressure overload on the ribosomal aggregation was seen already after a perfusion period of 60 min Earlier in vivo

studies (Fanburg and Posner 1968, Posner and Fanburg 1968) have shown that an increase in the labelling of RNA after injections of radioactive H_3PO_4 did not occur until 4 hr after the aortic constriction Although the experimental conditions are not exactly comparable the results of the present study suggest that the activity in the ribosomal cycle is affected earlier than the *de novo* synthesis of RNA after increased cardiac pressure work load An interesting finding in the studies referred to above was that no increase in the labelling of RNA in the heart occurred after aortic constriction if rats were given puromycin immediately after inducing the aortic constriction This might indicate the requirement of newly synthetized cytoplasmatic protein(s) for the current stimulation of the RNA synthesis

The mechanism for the stimulation of ribosomal aggregation in the heart by increased afterload are unknown These may involve changes in the activity of enzyme regulating the ribosomal initiation processes or alteration of levels of initiation factors Evidence has been presented indicating accelerated synthesis of mRNA after increased cardiac work load *in vitro* (Schreiber *et al* 1968) Changes in intracellular level of GTP could also be of significance since it has been shown that the activity of the aminoacyl tRNA transferase enzyme is dependent upon the intracellular level of GTP in the heart (Gibson and Harris 1972)

Increased pressure work load was recently found to accelerate the membrane transport of amino acids in the isolated rat heart (Ahren *et al* 1972) This effect could be of significance for regulation of heart protein synthesis since intracellular concentrations of free amino acids seem to be important for the charging of available tRNA (Novelli 1967) However it has also been shown that the intracellular levels of all amino acids are very constant when the rate of protein synthesis of the isolated rat heart is markedly changed (Hjalmarson *et al* 1970 Morgan *et al* 1971 a Jefferson *et al* 1971) In our opinion this does not rule out the possibility that changes in the membrane transport of amino acids can be of significance for the activity in the ribosomal cycle The finding of constant intracellular levels of all amino acid during extreme variations in the rate of heart protein synthesis might indicate that changes in rate of amino acid transport have taken place to preserve the intracellular concentration of all amino acids Objections can also be raised concerning the rationale of correlating the total intracellular concentration of amino acids to the rate of incorporation of radioactive amino acids into tissue proteins due to the postulated compartmentalization of the intracellular amino acid pools (Kipnis *et al* 1961 Kostyo 1964 Hider *et al* 1969 1971 a b)

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Methods

Mongrel dogs of both sexes were used They were anesthetized with an i v injection of thiopentone (30 mg/kg) both parotid glands were cannulated from the mouth and the maximal secretory rate which could be induced by pouring citric acid on the tongue of the animal was determined as described earlier (Holmberg 1971) The anesthesia was then deepened by an additional i v injection of the barbiturate an endotracheal tube was introduced and the anesthesia was maintained with ether In 5 dogs the auriculo-temporal nerve was exposed according to Burgen (1964) medial to the mandible and divided unilaterally on the mandibular joint whereas both this nerve and the nervous strands on the internal maxillary artery (Holmberg 1971) were severed unilaterally in 3 other dogs In 6 animals the latter operation was combined with division of the ipsilateral facial nerve just outside the stylomastoid foramen In another dog only the facial nerve was severed Maximal reflexly induced secretory rate was estimated on the 3rd—5th postoperative day in all the operated animals

Choline acetyltransferase determination

On the 7th postoperative day the animals (altogether 15 dogs) were killed by air embolism under thiopentone or chloralose anesthesia and the content of choline acetyltransferase was estimated in parotid glands and in one of the dogs in the facial nerves too

In another series of experiments 5 unoperated dogs were used to determine the choline acetyltransferase activity in normally innervated parotid glands

The parotid glands were carefully dissected the facial nerve was removed as thoroughly as possible and the auriculo-temporal nerve was divided close to the glandular parenchyma. After removal the glands were cleaned in saline and weighed The choline acetyltransferase activity was then determined according to the method devised by Hebb (Nordenfelt 1963 1965) Whole glands were used The acetone powder of the glands was made up in cysteine saline in a concentration of 50 mg/ml (the facial nerve in a concentration of 2? mg/ml) 0 4 ml of the parotid tissue extract (0 2 ml of the facial nerve) was incubated at 38 C for 1 h The acetyl choline formed was then estimated on the frog rectus and expressed in μg ACh/h/gland or μg ACh/h/g acetone powder

Activities of less than 10 μg ACh/h/g acetone powder of the parotid gland could not be measured

The wet weights of the denervated glands varied between 1 6 and 9 8 g The dry weights (acetone dried powders) were used when the denervated glands were compared with the corresponding contralateral glands Student s t test for paired values was used for statistical evaluation

Electrical stimulation of the facial nerve

Electrical stimulation of the facial nerve was carried out in 13 glands of 10 dogs In ? of the animals where both glands were used the tympanic nerve containing the preganglionic fibres to the gland was divided unilaterally in the middle ear as described by Heidenhain (1878) These operations were carried out 7 days before the acute experiment in order to cause a denervation supersensitivity in the glands For the acute experiment the animals were anesthetized with chloralose urethane (50 + 500 mg/kg) after induction with ether Tracheal cannula was inserted The parotid ducts were cannulated with polyethylene catheters which were connected to outflow bottles The drops from these bottles were recorded on a smoked drum with ordinate recorder or electromagnetic pens operated by photoelectric drop counters The facial nerve was exposed and divided as close to the stylomastoid foramen as possible It was stimulated through bipolar ring electrodes with square wave shocks of 2 ms duration 10—15 V intensity and a frequency of 20/s Stimulation was carried out for periods of 3—5 min with periods of rest for at least 5 min In some of the experiments stimulation of the facial nerve did not provok an flow of saliva at all Then a secretory response to this stimulus could often be produced after injection of eserine sulphate 3 μg or 30 μg in 0 3 ml of saline into the glandular duct using the method described by Emmelin Muren and Stromblad (1954) In some cases eserine produced a slow secretion but the nerve was not stimulated until this secretion had subsided Gallamine —4 mg/kg was given i v to 4 of the animals in order to abolish the facial contraction in response to stimulation of the facial nerve Artificial respiration was then given

Results

Choline acetyltransferase of normal parotid glands

The mean total enzyme activity in 5 normal dogs was 139 4 ± 10 0 (n = 10) μg ACh/h/gland This corresponds to a concentration of 287 ± 26 (n = 10) μg ACh/h/g

TABLE I Choline acetyltransferase activity in the partoid gland of the dog after section of the auriculo-temporal nerve 7 days earlier Mean ± S E are also given

Enzyme activity in μg ACh/h/whole gland Denervated	Contralateral	D/C	Enzyme activity in μg ACh/h/g acetone powder Denervated	Contralateral	D C	Reflexly induced secretion D C
20 8	74 0	28	50	160	31	64
13 2	105 5	12	40	300	13	80
56 8	234 6	24	45	1 0	26	71
84 0	143 2	59	240	400	60	64
32 1	148 1	22	40	200	20	64
41 4 ±13 0	142 0 ±27 0	29 ±8	83 ±39	246 ±46	30 ±8	69 ±3

acetone powder Right and left glands were found to differ very little thus the right glands were found to have a total activity and a concentration which was 98 ± 4 % (n = 5) and 102 ± 2 % (n = 5) respectively of that of the left glands

Choline acetyltransferase and reflexly induced secretion of denervated glands

As can be seen from Table I section of the auriculo-temporal nerve causes a great reduction of the choline acetyltransferase activity when estimated 7 days later The mean total activity of the denervated glands was found to be 29 ± 8 % (n = 5) of their control glands The concentration of the enzyme decreased to 30 ± 8 % (n = 5) Although the acetylcholine synthetizing power of the glands was diminished by about 2/3 of its normal capacity the reflexly induced secretion was only reduced by about 1/3

When the denervation included not only section of the auriculo-temporal nerve but also nerve fibres on the internal maxillary artery a more marked decrease in choline acetyltransferase activity was observed (Table II) After this operation the mean percentage figures for total activity and concentration were 9 ± 3 (n = 3) and 12 ± 3 (n = 3) respectively Reflexly induced secretion was abolished in these 3 glands

Although the facial nerve was always carefully dissected out from the gland it was suspected that twigs of the nerve escaped the dissection thereby contributing to the choline acetyltransferase activity The nerve trunk certainly contained choline acetyltransferase in one dog the part of the normal nerve which could be removed from the gland was found to have an activity of about 800 μg/ACh/h/g acetone powder In the corresponding part of the contralateral nerve which had been divided 7 days earlier the enzyme activity was not measurable

In 6 dogs division of the facial nerve was performed in addition to the 2 other nerve sections In only one out of these dogs a measurable quantity of acetylcholine was formed in parotid glands Thus 5 glands had a synthetizing power which was below the limit of detection i e less than 10 μg ACh/h/g acetone powder As shown in Table III 2 glands were able to secrete slightly when reflexly stimulated although they did not have a measurable enzyme content

TABLE II Choline acetyltransferase activity in the parotid gland of the dog after section of the auriculo-temporal nerve and nerve fibres on the internal maxillary artery 7 days earlier Mean ± S E are also given

Enzyme activity in µg ACh/h/whole gland Denervated	Contralateral	D/C	Enzyme activity in µg ACh/h/g acetone powder Denervated	Contralateral	D/C	Reflexly induced secretion D/C [a]
5 7	40 3	14	30	175	17	0
7 8	110 5	7	20	160	12	0
9 5	194 1	5	20	300	7	0
7 7	115 0	9	23	212	12	0
±1 1	±44 4	±3	±3	±44	±3	

In one dog only the facial nerve was cut in order to see if this could lead to an observable reduction in the otherwise intact gland, but no decrease was observed the mean percentage of total activity and concentration was 109 and 100 respectively

Gland weights

No significant decrease in dry weights (acetone dried powders) was observed after section of the auriculo temporal nerve alone However after the two more extensive operations the weight of denervated glands was diminished to about 80% of that of the contralateral ones ($p < 0.01$)

Electrical stimulation of the facial nerve

In one of the 2 glands where the tympanic nerve had been cut previously a large response 18 drops in 5 min was obtained but in all the other glands the response never exceeded 1 drop per min and was in most cases of the magnitude of 1 drop in 5 min Four of the 5 glands which did not secrete initially when the facial nerve was stimulated responded well to this stimulus when 3 or 30 µg of eserine had been administered through the parotid duct whereas the 5th one did not respond even after 300 µg of the drug The amounts of saliva obtained on facial nerve stimulation after eserine were very variable in some cases as much as 25 drops were obtained in 5 min

The secretory responses to stimulation of the facial nerve were mediated through a cholinergic mechanism because whether enhanced by eserine or not they were abolished by atropine 200 µg/kg i v Since they were also abolished after division of the facial nerve distal to the stimulating electrode the responses were not caused by irradiation from the electrode to the surrounding structures After Gallamine the secretion was only slightly reduced indicating that contraction of surrounding striated muscle contributed but little to the secretory responses It was not possible to assess with certainty on which side of the stimulating electrode the ganglion of the facial secretory pathway was situated the secretory response to stimulation of

TABLE III Choline acetyltransferase activity in the parotid gland of the dog after section of the auriculo-temporal nerve, nerve fibres on the internal maxillary artery and the facial nerve 7 days earlier. Mean ± S.E. are also given

Enzyme activity in µg ACh/h/whole gland Denervated	Contralateral	D/C %	Enzyme activity in µg ACh/h g acetone powder Denervated	Contralateral	D/C	Reflexly induced secretion D/C
0	106.9	0	0	225	0	0
7.3	140.9	5	10	150	7	0
0	133.1	0	0	150	0	0
0	186.5	0	0	150	0	5
0	153.3	0	0	225	0	6
0	169.9	0	0	225	0	0
1.2	148.4	1	2	188	1	2
±1.2	±11.5	±1	±2	±17	±1	±1

the facial nerve was about half as large after hexamethonium 20 mg/kg i.v. as before in 6 glands out of 8 studied, whereas it was unaffected in the other 2 glands.

Discussion

In the present study it has been found that like other salivary glands (Nordenfelt 1963, 1964, 1965) the dog's parotid contains choline acetyltransferase which disappears after division and degeneration of the cholinergic postganglionic nerves to the gland. Since all the enzyme measured with the method used thus seems to be contained in these nerves, the reduction of the activity of the enzyme has been used in the present work as an indication of the effectiveness of the denervation obtained after various operative procedures. Three sets of nerves were found to contribute to the activity of the choline acetyltransferase: the auriculo-temporal nerve, the fibres on the internal maxillary artery and the facial nerve. Each of these nerves probably contains different proportions of fibres beside the secretory ones, such as fibres to myoepithelial cells, striated muscle and vessels. It is likely that these kinds of fibres contain different amounts of choline acetyltransferase. Therefore the percentage activity of the enzyme remaining in the gland after a certain operation is probably not a very good quantitative index of the proportion of cholinergic secretory nerves left. Thus about 70 % of the normal reflexly induced secretory rate was still obtained when the auriculo-temporal nerve had been cut, but only some 30 % of the normal enzyme activity. This might partly be due to convergence of the nerves on the secretory cells and possibly also to supersensitivity (Holmberg 1971), but it is also possible that a large proportion of the enzyme which disappeared with the nerve was contained in other fibres than secretory ones. Correspondingly some of the 10 % of the normal enzyme activity which remained after section of both the auriculo-temporal nerve and the nerves on the internal maxillary artery and which vanished when also the facial nerve had been cut might well be contained in

somatomotor fibres in twigs of this nerve, which were difficult to dissect out from the gland It is evident that the proportion of secretory fibres in the facial nerve was very scanty electrical stimulation of it generally produced about 1 drop of saliva in 5 min whereas the secretory response to stimulation of the auriculo temporal nerve or the nerves on the internal maxillary artery is of a magnitude of about 30 drops of corresponding size per min (Holmberg 1971)

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Histochemical Studies of Uterine Innervation after Neurectomies

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Abstract

KANERVA L T MUSTONEN and H TERAVAINEN *Histochemical studies of uterine innervation after neurectomies* Acta physiol scand 1972 *86* 359—365

The distribution of nerves of the uterus containing acetylcholinesterase (AChE) and catecholamine (CA) was studied with light microscopic histochemical techniques after sympathectomy parasympathectomy and removal of the paracervical ganglion in the rat A rich plexus of AChE containing nerves was present both in the myometrium and around the uterine blood vessels Nerve fibres exhibiting CA fluorescence were abundant around the blood vessels but only sparse in the myometrium. Sympathectomy resulted in the disappearance of CA-containing perivascular nerve fibres but left intact the extravascular myometrial nerve fibres those containing CA and those containing AChE Parasympathectomy caused a slight decrease in the perivascular AChE-containing fibres On the other hand it had no effect on the myometrial AChE or CA-containing nerve fibres Bilateral destruction of the paracervical ganglion resulted in the complete disappearance of AChE- and CA-containing fibres both around the blood vessels and in the myometrium

The nerve fibres originating from the sympathetic hypogastric plexus and the sacral parasympathetic nerves converge on the paracervical region of the uterus where a ganglion is to be found (Frankenhauser 1867 *cf* also Krantz 1959) Both types of nerve fibres synapse on the neurones of the paracervical ganglion (Mustonen and Teravainen 1971) although parasympathetic stimulation does not elicit action potentials in the uterus (Bower 1966)

Two kinds of nerve fibres can be histochemically distinguished in the uterus acetylcholinesterase-containing (cholinergic) fibres (Coupland 1962) and catecholamine-containing (adrenergic) nerve fibres (Owman and Sjöberg 1966) The two nerve fibre types are visible in the myometrium and around the blood vessels

The present study was made to find out to what extent the uterus receives its innervation directly from the lumbar sympathetic and the sacral parasympathetic nerves and/or from the neurones of the paracervical ganglion

Material and Methods

Operative procedures Altogether 29 operated and unoperated adult Sprague Dawley female rats in diestrus (Long and Evans 1922) were analyzed in the present experiments Parasympathectomy was carried out on 5 of the rats either by cutting the sacral nerve roots or by intraspinal injection of a small amount of 2 5 % glutaraldehyde in the region of the cauda equina The successful parasympathectomy was confirmed by urinary rentention The uterus was examined 48 to 52 h after the operation Sympathectomy was carried out on 12 of th rats by carefully coagulating all the nerves and ganglia from both sides of the aorta from the coeliac ganglion to about half way along the internal iliac artery Sympathectomy resulted in some hyperemia of the uterus The uterus was removed 7 or 14 days after the operation The paracervical ganglion of 12 rats was bilaterally coagulated with a hot iron avoiding damage to the blood essels and the uterus examined 2 7 and 14 days after the operation All the operations were made under ether anesthesia One half of the uterus was used for the demonstration of cholinesterases and the other for catecholamine fluorescence

Demonstration of cholinesterase activity The uterus was fixed with 3 5 % neutralized formaldehyde immediately after removal at 4 C for 6 to 12 h and washed for about 3 h in distilled water The specimens were sectioned frozen at 15—30 mμ and used free floating throughout the histochemical procedure Acetylcholinesterase (AChE E C 3 1 1 7) activity was demonstrated with acetylthiocholine iodide (Fluka AG Buchs) as a substrate and 10^{-5} M iso OMPA (tetra isopropylpyrophosphoramide L Light & Co Ltd Colnbrook) to inhibit other (non specific) cholinesterases (ns ChE E C 3 1 1 8) Butyrylthiocholine iodide (Fluka AG Buchs) was used together with 10^{-5} M 284C51 to demonstrate selectively ns ChE activity The method used was based on the Gomori modification (1952) of the Koelle (1951) technique

Aromatic monoamines Catecholamines were demonstrated with the aid of formaldehyde induced fluorescence (cf Eranko 1967 a) The specimens were dropped in liquid nitrogen or propane precooled by liquid nitrogen and dried in vacuo for 5—6 days at about −40 C exposed for 1 to 2 h at 80 C to paraformaldehyde gas equilibrated to within 40 % relative humidity and embedded in paraffin Fluorescence microscopy was carried out on 5 μ sections deparaffinized in xylene with a Wild microscope using mercury lamp HBO 200 and Schott BG 12 BG 38 and two OG 1 filters

Results and Discussion

Several acetylcholinesterase (AChE) containing nerve fibres are present in the nerve plexus running in both the circular and the longitudinal muscle layers of the uterus as well as around the blood vessels in unoperated animals (Fig 1) Only a few fibres extend into the basal subendothelial layers apart from around the glands in the cervix (Fig 2) As Adham and Schenk (1969) pointed out the nerve net is

Fig 1 Acetylcholinesterase (AChE) activity in the muscular layer of the uterine horn of untreated rat AChE activity in both myometrial (arrow) and vascular (crossed arrow) nerve fibres × 400

Fig 2 AChE activity in the cervix of untreated rat AChE activity in possible sensory endings (arrow) around the myometrial glands (B) × 400

Fig 3 AChE activity 7 days after sympathectomy The activity is left in both the myometrial and the vascular nerve fibr Cervix uteri × 400

Fig 4 AChE activity 7 days after the parasympathectomy The activity can be seen in both the myometrial (arrow) and the vascular (crossed arrow) nerve fibres A slight decrease is noticeable in the number of vascular fibres Cornu uteri × 200

Fig 5 The AChE activity after bilateral paracervical ganglionectomy demonstrates the complete disappearance of AChE containing nerve fibres Cornu uteri × 200

Fig 6 Nonspecific cholinesterase after bilateral paracervial ganglionectomy from the same site as shown in Fig 5 The Schwann cells devoid of axons arrow and the perimetrium (P) show strong activity × 200


G
1
2
3
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6

most dense at the cervix. The activity of non specific cholinesterases (ns ChE) follows the distribution of AChE activity, but is also intense around the glands and sensory endings in the perimetrial coat of the uterus (Fig 6)

Neither sympathectomy (Fig 3) nor parasympathectomy (Fig 4) has a clear effect on the distribution of AChE containing (cholinergic for ref see Eränko 1967 b) nerve fibres. However, comparison with the unoperated uteri shows that parasympathectomy causes a slight decrease in the number of perivascular AChE positive nerves. Bilateral destruction of the paracervical ganglion contrasts markedly with these observations since complete disappearance of AChE containing nerve fibres from both the myometrium and the perivascular space can be observed after the operation (Fig 5). The previous course of the nerves can still be seen after staining for ns ChE. This glial enzyme thus shows the Schwann cells devoid of the axons (Fig 6) indicating that the disappearance of AChE is due to the axon degeneration

The nerve fibres exhibiting catecholamine fluorescence are mostly confined to the vascular nerve plexus (Fig 7), but a few extracellular varicose nerves (Fig 8) are found especially in the cervical region as Adham and Schenk (1969) have reported. The presence of extravascular adrenergic nerves in the rat has previously been denied (Norberg and Fredricsson 1966 Sjoberg 1967) although they are found in several other mammals (Sjoberg 1967). These myometrial catecholamine containing fibres disappear only after the destruction of the paracervical ganglion but not after sympathectomy or parasympathectomy. The perivascular catecholamine containing nerves disappear both after the gangliectomy and after the sympathectomy (Fig 9). Parasympathectomy has no effect on the number of fluorescing fibres

The results show that all nerve fibres pass either through or close to the paracervical ganglion because bilateral electrocoagulation of the paracervical ganglion results in total degeneration of the uterine nerves. Preganglionic sympathetic denervation causes degeneration of the perivascular catecholamine containing nerves indicating that these nerves originate from the lumbar sympathetic ganglia. Since the myometrial nerves degenerate only after the destruction of the paracervical ganglion their cell bodies are most probably located in the ganglion. This ganglion has been previously shown to contain catecholamine neurons in the rat (Kanerva 1971 Hervonen *et al* 1972 b Kanerva *et al* 1972) as in other mammals studied (Owman *et al* 1967 Rosengren and Sjoberg 1967 Furness and Malmfors 1971)

It is evident that the myometrium of the rat is only slightly innervated by adrenergic nerves compared with the results obtained from other mammals (Sjoberg 1967). The cat which has rich adrenergic innervation in the uterus may receive myometrial adrenergic fibres not only from the paracervical ganglion but also from the inferior mesenteric ganglion (Rosengren and Sjoberg 1967). None of the myometrial adrenergic nerve fibres seems to originate from the hypogastric nerves in the rabbit and the guinea pig (Sjoberg 1967). The sparce myometrial adrenergic innervation in the rat does not necessarily make the contribution of catecholamines to the function of the rat uterus unlikely since it is possible that other mechanisms

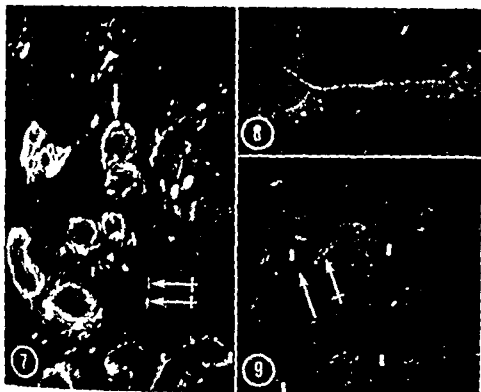


Fig 7 Formaldehyde induced fluorescence (FIF) in the cervix of the uterus Strong FIF in the vascular nerves (arrow) and few extravascular nerve fibres (crossed arrow) × 200

Fig 8 Extravascular varicotic nerve fibre in the cervix of the uterus × 400

Fig 9 FIF 7 days after sympathectomy The perivascular FIF (arrow) has completely disappeared but a few extravascular fluorescent nerves are visible (crossed arrow) around the blood vessel (B) × 200

exist to control the uterine catecholamine concentration for example by humoral means (cf Kanerva and Teravainen 1972)

General agreement has not been reached concerning the functional importance of the sacral parasympathetic innervation of the myometrium The most widely accepted opinion seems to be that the sacral nerves do not directly innervate the smooth muscle of the uterus (e g Bower 1966) Most AChE containing nerves originate from the paracervical ganglion since they degenerate after its destruction The nerve fibres remain intact after preganglionic denervation of the parasympathetic nerve fibres Sympathectomy also causes no changes in the acetylcholinesterase reaction localized in the myometrium nerves Physiological studies have found that preganglionic parasympathetic stimulation can cause contraction of the uterus while other reports claim that the sacral nerves have an inhibitory effect on the uterus (for ref see Gruber 1933 Sjoberg 1967) On the basis of

present findings and the contraversial electrophysiological results, the present authors are inclined to claim that the AChE containing fibres visible in the myometrium originate from the paracervical ganglion and contribute directly to the innervation of the uterus Whether some of the AChE containing nerve fibres are sensory in nature or represent adrenergic fibres containing cholinesterase activity at their membrane (Jacobowitz and Koelle 1965 Hervonen *et al* 1972 a) remains unsolved

On the basis of the present histochemical findings it can be concluded that the myometrium receives most of its cholinergic nerve fibres directly from the para cervical ganglion This ganglion is also the site of the synapse between pre and post ganglionic parasympathetic nerve fibres This finding explains the lack of changes in the acetylcholinesterase reaction seen in myometrial nerves after pre ganglionic denervation On the other hand the sympathetic system seems to run into the myometrium either from the paracervical ganglion (short adrenergic neurons) or from more remotely located sympathetic ganglia (long adrenergic neurons)

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studied in response to natural stimuli which are controllable in terms of amplitude and time course and the responses can be quantitatively recorded both in man and in nonanesthetized animals Quantitative studies of this reflex system may thus contribute general information on properties of polysynaptic reflex systems

Earlier studies of the time course of the middle ear reflex responses in animals have shown that the reflex responses are not generated by a linear system Latency rate of rise and frequency of oscillation have been measured in acute experiments on anesthetized or decorticated rabbits (Lorente de No 1933 1935, Wersall 1958) These experiments have been performed on the closed loop system *i e* when the muscle contraction attenuates the stimulus Since nonlinear properties are involved it is difficult to draw conclusions on the reflex mechanism itself from data obtained in the closed loop system The same difficulties are inherent in results from human experiments (Møller 1962 Dallos 1964 a 1964 b) In experiments on nonanesthetized rabbits with the middle ear muscles inactivated on one side the open loop system has recently been studied qualitatively The system was found to be nonlinear with regard to amplitude and to direction of change of stimulus amplitude (Borg 1972 d) rendering quantitative analysis and modelling difficult

The aim of the present investigation was to study quantitatively the open loop characteristics of the total middle ear reflex, *i e* m stapedius and m tensor tympani in conjunction and of the isolated m tensor tympani reflex and to obtain a mathematical description of the system The emphasis was laid on the onset characteristics in response to tone bursts which were regarded most important for the control of sound transmission The reflexes were treated as stepwise linear systems which were analyzed with linear methods The open loop characteristics could be described by a second-order transfer function with a transport delay and with intensity and direction dependent parameters

Methods

As a basis for the description of the methods of analysis the middle ear reflex system is shown as a block diagram in Fig 1 Normally the middle ear reflexes work as a closed loop system *i e* a muscle contraction influences the vibration of the ossicles of the middle ear and decreases the transmission of the stimulus sound to the cochlea The influence on ossicular vibration gives rise to a change of the impedance of the middle ear that can be used to measure the muscle activity (see below) The change in transmission at a low frequency (0 8 kHz) is proportional to the change in impedance except near maximum impedance change (Borg 1972 a) Sound stimulus (input signal) in one ear gives rise to muscle contraction in both ears (feed back signals) The time course of the ipsilateral and the contralateral muscle contraction cannot be expected to be identical since the crossed reflex arc is longer than the ipsilateral one and contains one additional neuronal link (Borg 1972 f) The feed back signal is multiplied by the input in such a way that an increase in input sound level of 1 0 dB gives about a 0 dB increase in actual input to the cochlea (actuating signal) *i e* the closed loop efficiency of regulation is 0 7 dB/dB (Borg 197 d) If the input is kept in logarithmic units this interaction is a summation The efficiency of regulation for the open loop system at steady state is equivalent to about ? dB decrease per 1 dB increase in stimulus intensity For linear units the attenuation of a 0 5 kHz tone (in dyn/cm^2) has been found to be close to a straight line as a function of sound pressure (in dyn/cm Borg 197? d) This indicates that the interaction can be approximated by a summation also when linear units are used The open loop gain has been estimated to 4 (Borg 1972 d) High frequency sound (?0 kHz) is influenced only by middle ear muscle activity well above reflex threshold but above this level the efficiency of regulation is the equivalent to that of 0 5 kHz (Borg 1972 d)

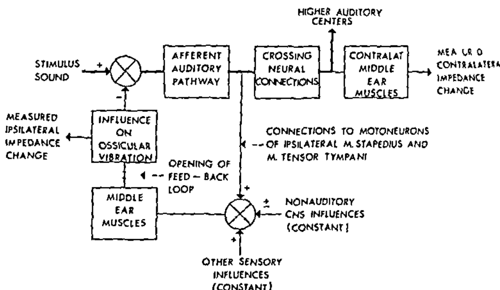


Fig 1 Block diagram of acoustic middle ear reflex system Tone bursts in one ear elicits muscle contractions bilaterally that are measured as changes of the acoustic impedance of the ears Ipsilateral closed loop system contains the path Stimulus sound Afferent auditory pathway "Connections to middle ear muscle motoneurons Middle ear muscles The feed back signal interacts with the input signal through Influence on ossicular vibration Other sensory influences and influences from the central nervous system (CNS) were assumed to be constant or absent The loop is opened by cutting and denervating the middle ear muscles in the stimulated ear (broken lines) The open loop system experimentally studied is that of the crossed reflex which differs from the ipsilateral system by th Crossing neural connections

The goal of the present study was to determine the open loop transfer properties for the ipsilateral reflex Thus the feed back loop was opened in the stimulus ear (broken lines of Fig 1) which at the same time abolished the possibility of measuring the ipsilateral reflex re ponses The properties of this reflex could however be obtained from the crossed reflex if the properties of the crossing neural connections were known (Fig 1) These could be obtained by comparing simultaneously recorded responses of the ipsilateral and contralateral reflex under closed loop conditions

The middle ear reflexes have nonlinear properties (Lorente de Nó 1933 1935 Moll r 1962 Dallos 1964 a 1964 b Borg 1971 1972 d) which means that the linear analysis methods can be applied only under those conditions in which the system behaves approxima el linearly Linear conditions for the middle ear reflexes can be a sessed on the ba is of results from earlier experiments (Borg 1971) In these experiments evidence was presented that the middl ear reflexes can be considered as consisting of a number of functional components working in parallel These components are not necessarily identical to motor units (Borg 1971) and are therefore called reflex units They have different thresholds and the time course of such a unit is faster as the threshold becomes higher Each reflex unit is as umed to co tribute to onl a fraction of the total response and to function as a linear system of the same order The number of parallel reflex unis that are active in each stimulus situation is determined by the amplitude of the signal The part of the system that is active is thus dependent on the signal amplitude For step impulse and other rapid rise and fall time stimuli all the units that are activated are triggered nearly simultaneously (see further Borg 1971 e) Th the response of the system as defined at each amplitude will respond to rapidly rising or falling stimul in the same manner as a system composed of a number of elements in p rall) whose thresholds need not be considered For such a system linear analysis methods can be us d If slow ri and/or fall time stimuli are used the system cann t be approximated equally well to a linear s tem s nce the various units are triggered successivel at different times

Recording of the reflex responses

The responses of the acoustic middle ear reflexes were recorded simultaneously in both ears as changes in the acoustic impedance of the ears at 800 Hz The measuring devices were hermetically sealed in the ear canals Each of the two devices contained three electroacoustic transducers one stimulus earphone one earphone for generating the 800 Hz measuring tone and one microphone The measuring tone (explorer tone) had an intensity at the eardrum of 65—70 dB SPL (re 0 0002 dyn/cm²) The output of the microphone was balanced out electrically Contractions of the middle ear muscles upset the balance and the resulting 800 Hz signal were recorded on a two-track tape recorder together with a 7 4 kHz trigger signal which was synchronous with the stimulus

The steady state amplitude of the measured impedance change is approximately proportional to the force developed by the m stapedius (Borg 1972 a) The time course of the impedance change is equal to the time course of the contraction of the muscles since the middle ear s resonant frequency is far above that expected for the reflex (Møller 1965) Thus impedance change can be expected to be an accurate measure of middle ear muscle activity For further details of the method see Møller (1961) and Borg (1972 a 1972 b)

Twelve rabbits from a group of 78 animals whose reflex responses were previously recorded (Borg 1972 c 1972 d) were selected for the analysis of the dynamic properties of the middle ear reflex Ipsilateral and contralateral closed loop responses of the total reflex were recorded and analysed in five nonoperated rabbits Isolated m stapedius reflex responses were recorded only ipsilaterally after tendotomy of m tensor tympani in the stimulus ear (one rabbit) The open loop responses of the total reflex were recorded contralaterally in three rabbits (see Fig 1) after inactivation of both muscles in the stimulus ear (in one case only the m stapedius was inactivated) The responses of the isolated m tensor tympani reflex were obtained ipsilaterally and contralaterally in three rabbits after bilateral denervation of the m stapedius by extraction of the n facialis through the stylomastoid foramen The open loop responses of the m tensor tympani reflex were recorded contralaterally in one additional case after also cutting the m tensor tympani in the stimulated ear Since the m tensor tympani has not been found to influence sound transmission in the intensity range below about 120 dB SPL at 2 0 kHz (Borg 1972 d) it was expected that the open loop characteristics would be obtained even though the m tensor tympani was intact in the stimulated ear This was verified by the results except for two cases (out of 18 rabbits with m stapedius paralysis) where oscillations appeared in the responses indicating an attenuation of the stimulus sound

Test stimuli

Most methods for dynamic analysis have been developed for study of linear systems In such a system the response to the sum of two stimuli is equal to the sum of the responses when the two stimuli are presented separately The properties of a linear system are completely described by the response to stimuli containing all frequencies (*e g* broadband noise impulse stimuli sinusoids in a wide frequency range) Thus the response to a brief impulse excitation contains the total information about the dynamic properties of a linear system and can be used as the basis for calculating the frequency transfer function of the system Since the step function is the integral of the impulse the responses to step stimuli can be used in the same way as the impulse response for calculation of frequency transfer functions if preceded by a differentiation If the system is nonlinear in the way that it has different onset and decay properties (as do the middle ear reflexes) positive and negative step stimuli ought to be used in order to separate onset and decay properties

The onset properties can be supposed to be most important for the role of the muscle in regulation of natural input to the cochlea *e g* for fast attenuation of potentially overloading sound If the impulse properties approximate the onset properties — contrary to what can be expected due to the direction dependent properties — use of the impulse could facilitate determination of the onset open loop properties of the middle ear reflex in man without the need for surgery In human experiments transfer properties obtained from impulse responses and the onset of step modulated 1450 Hz pure tones have been found to differ (Møller 1962) This could however have been due to the fact that sound is indeed attenuated at 1450 Hz (Borg 1968) and that the open loop properties were not obtained Thus in the present study impulse stimuli were used to investigate the possibility of the approximating open loop onset conditions

The step stimuli were 1 0 s bursts of pure tones of 2 0 kHz and 0 5 kHz (rise time to 90 % and decay time to 10 % both 2 ms) in the intensity range 80 to 120 dB SPL The stimulus artefacts were much more prominent at 0 5 kHz than at 2 0 kHz which greatly decreased the number of experiments that were suited for quantitative analysis of the temporal characteristics at 0 5 kHz stimulus frequency Furthermore 16 ms bursts of a 2 0 kHz pure tone were used to approximate impulse stimuli

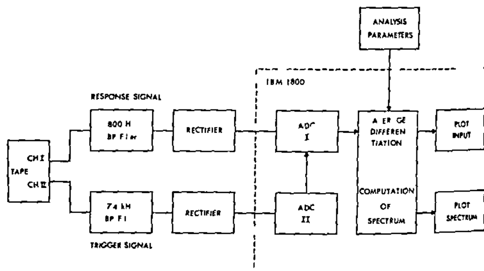


Fig 2 Signal handling by the analysis system Trigger signal 7 4 kHz pure tone from channel II (CH II) on tape recorder (Revox G 35) via bandpass filtering and rectification to analogue input of computer (IBM 1800) to analogue to digital conversion (ADC) Response signal 800 Hz pure tone from channel I (CH I) via bandpass filtering and rectification to ADC I which is started when trigger level is reached in ADC II

Analysis methods

The aim of the quantitative analysis was to describe the properties of the acoustic middle ear reflex in mathematical terms This was achieved in four steps (1) Rectified and smoothed impedance change signals in the time domain were transformed to the frequency domain (Fourier transform) (2) The order of differential equation describing the system was determined from the steepness of the high frequency flank of the amplitude response and the high frequency asymptote of phase response (3) Functions were synthetized on the basis of the Fourier transforms and approximated to the experimental spectra according to a least square criterion and (4) The intensity dependence of the parameters was determined and separate functions for onset and decay properties were synthetized

The analysis was based on Fourier transform of analog to-digital converted samples of the signal representing the reflex response The start of the sampling was synchronized with the start of the stimulus by means of the 7 4 kHz trigger signal

The principles of the analysis system and the analog signal processing are shown as a block diagram in Fig 2 The amplitude modulated 800 Hz response signal representing the middle ear muscle activity was bandpass filtered (center frequency 800 Hz bandwidth 100 Hz) rectified lowpass filtered (cut off at 50 Hz slope of 18 dB/octave) and fed into one of the analog inputs of the computer (IBM 1800 The trigger signal (7 4 kHz) was bandpass filtered, rectified lowpass filtered cut off at 200 Hz slope of 18 dB/octave) and fed into another analog input A simplified flow chart of the data processing is sho n in Fig 3 The trigger signal channel was contin [illegible] ly sampled at 1 25 ms interval When a trigger signal arr ed, sampling and analog to-dig t l convertion (ADC) of the response signal was initiated The sampling of the response in [illegible] ideal case should start simultaneously with the stimulus in the method used the error wa [illegible] than 1 25 ms

A spectral resolution [illegible] 1 0 Hz was regarded as sufficient to describe the refe system A total analysis tim [illegible] of 800 ms was chosen which gives a spectral resolution of 0 625 Hz (1/2T) Since th [illegible] fl x system was expected to have a resonance in the region of 20 Hz (see *e g* Borg 197 [illegible] quist folding frequency (FN) of 50 Hz was regarded a sufficient This implies a s [illegible] pling interval of 10 ms (1/ FN see further *e g* Blackman and Tukey 1958) T [illegible] lem f alia ing was reduced by the lowpass filtering (50 Hz see above of the respon [illegible] It [illegible] and that even the best recordings gave an insufficient signal to noise ratio m [illegible] high f equency p rt f the computed spectrum unstable when calcula tions were b [illegible] gle recordings To o e ome this difficulty 10–40 respor es were averaged befo [illegible] ra were calculated Th averaged input signals were plotted f r visual

checking After differentiation in the case of step response the time function was subjected to Fourier transform and the computed Fourier amplitudes was smoothed by "hanning" (see Blackman and Tukey 1958) Impulse responses gave better statistical stability at high spectral frequency and were used for the determination of direction of phase asymptote (see Fig 6)

The dynamical properties of the recording and analysis system

The electrical filtering of the recorded impedance change contributed to the computed Fourier amplitude and phase values This was compensated for by correction of the derived frequency transfer function The transfer characteristics of the recording and analysis system were obtained by Fourier transform of the differentiated response of the system to a step function modulated 800 Hz signal that had been passed through the total recording and analysis system. The average response to ten such signals was analysed The corrected reflex transfer functions were then obtained by subtracting the amplitude values (in dB) and the phase values The corrected properties are shown in the figures and served as the basis for the determination of the mathematical transfer functions

Results

The results will be presented below in three main sections (a) the total reflex (b) the isolated m stapedius reflex and (c) the isolated m tensor tympani reflex The closed loop and the open loop properties of each of the three systems will be described A separate analysis of the onset and decay properties will be presented for the total reflex and the isolated m tensor tympani reflex as well as a mathematical model of their dynamic properties The isolated m stapedius reflex will be treated only briefly since it was found to be closely similar to the total reflex.

A *The total reflex*

Onset of step response of closed loop system The closed loop form is the normal status of the system It is more complex than the open loop system and therefore is not as suitable for analysis To assess the properties of the ipsilateral open loop system the characteristics of the crossing neural connections (Fig 1) first must be known These characteristics can be obtained most easily from the relationship that exists between the simultaneously recorded ipsilateral and contralateral responses of the closed loop system

Fig 4 shows the amplitude response (left graphs) and the corresponding phase shift (right graphs) of the ipsilateral (upper row) and contralateral (middle row) total middle ear reflex. The ipsilateral and contralateral responses were recorded simultaneously in response to stimulation with 2 0 kHz pure tones at three intensities 102 dB SPL (solid line) 98 dB SPL (dashed line) and 94 dB SPL (dotted line) The graphs at the left of Fig 4 show that the closed loop system had lowpass characteristics The upper cut off frequency (point of 3 dB attenuation) increased from 14 0 to 20 5 Hz as intensity was raised from 94 to 102 dB SPL A narrow resonance peak occurred at 19 Hz for the highest intensity and there was also a second resonance peak at 38 Hz which was most likely a distorsion product of the 19 Hz oscillation generated in the feed back loop The height of the main peak as well as its frequency decreased as the stimulus intensity was decreased The frequency of the main peak is consistent with the oscillations that have been observed in time course recordings of the reflexes (Borg 1972 c) The majority of the animals showed less oscillations than the case illustrated in Fig 4

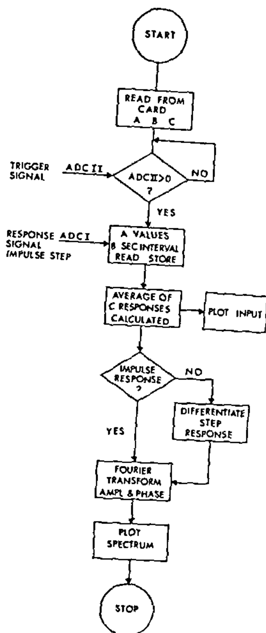


Fig 3 Flow chart er data processing and calculation to Fourier transform

The graphs at the right of Fig 4 show that the phase characteristics were also intensity dependent The phase shift decrea ed as sound level was increased except at resonance where phase shifted rapidly at frequncies near the peak

To determine the transfer characteristics of the crossing neural connections"

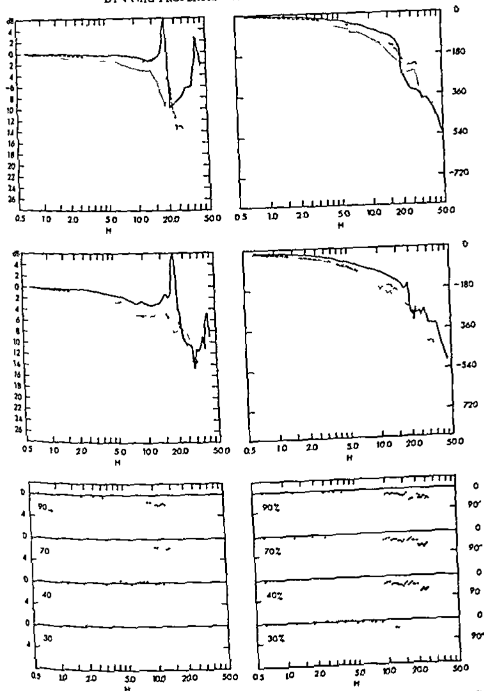


Fig 4 Transfer functions calculated from [illegible] (upper row) and simultaneously recorded [illegible] lateral reflex [illegible] row). Stimulus 2.0 kHz [illegible] 102 dB SPL 97 [illegible] of maximal [illegible] change [illegible] 94 dB SPL 86 [illegible] Bottom graphs [illegible] difference between [illegible] and contralateral transfer functions [illegible] at four intensity levels corresponding to approximately 90 70 40 and 30 [illegible] in each case [illegible] 4 animals.

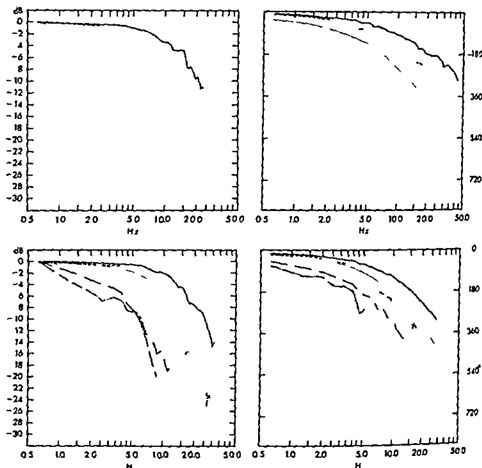


Fig. 5. Transfer functions for onset of the open loop response to contralateral stimulation with 2.0 kHz bursts. Upper graphs: m. stapedius and m. tensor tympani paralysis in stimulus ear. ——— 112 dB SPL, 99 %; – – – 102 dB SPL, 89 %; – – – 94 dB SPL, 50 %. Lower graphs: the same animals as in Fig. 4, m. stapedius paralysis in stimulus ear. ——— 107 dB SPL, 95 % of maximal response; – – – 101 dB SPL, 93 %; – – – 97 dB SPL, 86 %; – – – 93 dB SPL, 78 %; — — 89 dB SPL, 50 %; – – – 85 dB SPL, 23 %.

(Fig. 1), the difference between the transfer functions based on the simultaneously recorded ipsilateral and contralateral total reflex responses were calculated. The bottom graphs of Fig. 4 show averaged differences (contralateral-ipsilateral) at four relative response amplitudes for four animals. The crossing connections were found to have a low pass characteristic that was more marked the higher the stimulus intensity.

Onset step response of open loop system. The ipsilateral reflex system could not be studied experimentally under open loop conditions. Recordings of the impedance change in the contralateral ear were obtained after denervating the muscles of the stimulated ear. Thereafter, the ipsilateral open loop characteristics were obtained from the crossed reflex data by subtracting the amplitude and phase values of the crossing neural connections from those obtained for the crossed reflex. Fig. 5 shows

amplitude (left) and phase (right) properties of responses to tonebursts of 2 0 kHz at various intensities The upper graphs are based on one animal wherein both the m stapedius and m tensor tympani were inactivated in the stimulus ear At the stimulus frequency used (2 0 kHz) it was assumed that a satisfactory approximation to the open loop situation was reached after denervating m stapedius and leaving the m tensor tympani intact (see Methods) The transfer functions presented in the lower graph of Fig 5 were obtained in another rabbit under such conditions The closed loop properties from this same animal are shown in Fig 4 The open loop system in both cases is seen to have a smooth low pass transfer function The peaks at 19 and 38 Hz in the closed loop transfer functions shown in Fig 4 do not appear in Fig 5 as a result of denervating the m stapedius On the other hand increased sound intensity is still seen to have a prominent effect on the cut off frequency (point of 3 dB attenuation)

In the lower graph of Fig 5 it is seen that the cut-off frequency increased from 1 1 to 11 9 Hz as the intensity was increased from 85 to 107 dB SPL These intensities resulted in responses from 23 % to 95 % of maximal amplitude of the recorded impedance change The results from the animal in which both muscles were inactivated in the stimulus ear (Fig 5 upper graphs) showed a similar dependence on stimulus intensity The high frequency slope of the amplitude response was approximately 12 dB/octave in both cases

The phase shift is shown in the right part of Fig 5 It was found to decrease at increasing sound levels This decrease is expected on the basis of the intensity dependent change in the amplitude transfer functions However the phase shift did not reach a horizontal asymptote as expected for a low pass system These deviations are probably an effect of the transport delay which is known to result in a phase shift proportional to the frequency

Determinating the frequency at which the phase shift reaches 180° is of special interest since a corresponding (linear) closed loop feed back system will show a tendency to oscillate at this frequency if the gain is sufficiently high As shown in Fig 5 the frequency at which the phase shift was 180° increased from 7 2 to 20 6 Hz (upper graph) and from 4 0 to 15 6 Hz (lower graph) as the stimulus intensity was increased These frequency values were determined for the crossed reflex system The corresponding values for the ipsilateral reflex system could be calculated on the basis of the difference between the ipsilateral and the contralateral reflexes *e g* as shown in Fig 4 (bottom graph) The frequency for which the phase shift was 180° then fell between 6 0 Hz and 21 0 Hz for the ipsilateral reflex of the case shown in Fig 5 (lower graph) This is close to the resonant frequency of the closed loop system (19 Hz Fig 4)

Mathematical description of the open loop transfer functions If the assumption of linearity (see Methods) is valid it should be possible to approximate the Fourier transform of a differential equation to the calculated amplitude and phase values This differential equation should be of the same order for all intensities Only the constants would be allowed to vary

The order of the differential equation to be used for the approximation can be derived from the slope of the high frequency flank of the amplitude transfer function or from the

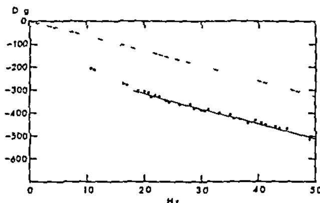


Fig 6 Phase shift of impulse response (dots) plotted on a linear frequency scale Direction of asymptote determined by fitting straight line to the segment 20 0 to 50 0 Hz (solid line) y = −6.57X − 18? Transport delay phase shift is shown by broken line y = −6.57X

zontal asymptote of the phase shift at high frequencies. The slope of the amplitude transfer function of 12 dB/octave (Fig 5) tends to suggest a second order system The corresponding phase shift should have an asymptotic value of 180 The measured phase values however also include the transport delay and therefore the phase angle of the transfer function is expected to show a constant slope at high frequency The asymptote of the phase shift of the transfer function can thus be determined after subtracting the phase shift due to the transport delay from the originally calculated values The transport delay gives rise to a phase shift $F = e^{-tj\omega}$ (t = delay in s ; ω = complex frequency) which is a straight line having a negative slope proportional to the delay

Fig 6 shows the phase shift determined from the impulse response and plotted on a linear frequency scale (dots) The impulse response was used for this determination since the statistical stability of the phase values at high frequency was better than for differentiated steps. The straight continuous line of Fig 6 shows the least mean square error fit to the measured values (dots) in the frequency range from 20 0 Hz to 50 0 Hz The slope was found to correspond to a delay of 18 ms which is shorter than the latency measured from the time response directly which was about 35 ms This deviation is accounted for by the smoothing of the filters that was compensated for in the frequency transfer function but not in the time domain The latency based on uncorrected phase values was 38 ms which on the other hand is in close agreement with the value in the time domain The phase shift due to the delay alone is shown by the straight broken line through the origo The difference between the total phase shift and the phase shift due to the delay is seen to be close to 180 and thus suggests a second-order system with a transport delay as a mathematical model of the reflex

The experimentally derived open loop frequency transfer function was thus described by a mathematical model of the form $G_0(j\omega) = \frac{K e^{-t_1 j\omega}}{(j\omega + a)(j\omega + b)}$ where K, t_1, a, b are parameters and jω is the complex frequency K including steady state open loop gain is in these calculation an arbitrary constant It has been shown earlier to have a value of approximately 4 (for 0 5 kHz sound Borg 1972 d) The constants t_2 and t_3 were first determined by approximation of the computed amplitude transfer function of the model to the experimentally derived values The transport delay (t_1) is without influence on the amplitude properties Thereafter t_1 was determined from the phase values The amplitude value of the calculated function was set equal to the experimental value for the lowest frequency (0 6[illegible] Hz) The least mean square error was used as the criterion for the best fit between the theoretical function and the experimentally derived values

Fig 7 shows one example (shown earlier as the dash dotted line in Fig 5 lower graph) of experimentally derived values (dots) and the fitted function The agreement is seen to be good both for amplitude and phase characteristics The small peaks at high frequencies (about 30 Hz) were regarded without importance for the function of the system and were not incorporated in the model In general the fit was found to be best if the two parameters a and b were equal

The lower graph of Fig 7 shows the intensity dependence of the parameters t_1

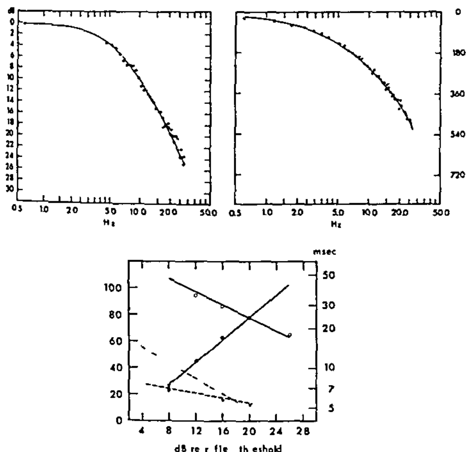


Fig 7 Upper graphs second order system frequency transfer function (solid line adapted to amplitude and phase parameters calculated from onset of open loop response (dots). Lower graph values of parameters as a function of stimulus intensity (in dB re reflex threshold). Onset is shown by open and filled circles, decay is shown by open and filled triangles. Filled symbols show a (= b) (left scale 1/s), open symbols show logarithm of latency (t_1 right scale).

a and b. a and b are equal and are shown as filled circles. They are seen to vary approximately as linear functions of sound intensity (in dB re reflex threshold the continuous line). The logarithm of t_1 (open circle) was approximated by a linear function of stimulus sound intensity (in dB the continuous line). Thus it is possible to synthetize a transfer function with intensity dependent parameters $G(j\omega) = \frac{K e^{-t_1 j\omega}}{(j\omega+a)(j\omega+b)}$, $\log t_1 = -0.024x - 1.13$, $a = b = 4.28x - 7.1$, x is intensity in dB re reflex threshold. A large value of the parameters a and b indicates a fast response. According to a linear intensity dependence a and b should be negative at threshold. Since this evidently cannot be the case it must be assumed that the relationship deviates from linearity at low sound level. The broken lines of the lower graph refer to the decay of step response from the same animal (see next section).

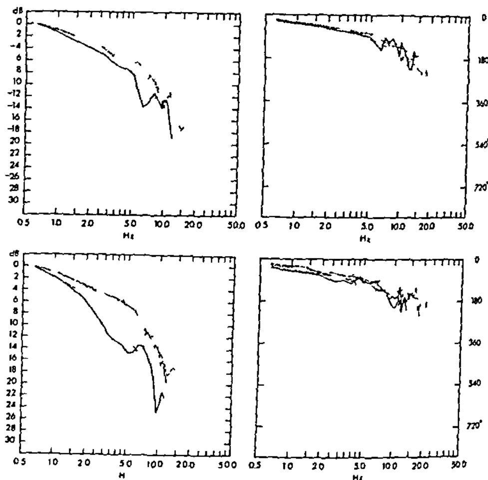


Fig. 8. Frequency transfer function calculated from decay of step response of closed loop system (upper graphs) and that of open loop system (lower graphs). The same animal as in Fig. 4 and Fig. 5. Upper graphs ——— 108 dB SPL, 102 dB SPL, 98 dB SPL, 94 dB SPL. Lower graphs ——— 101 dB SPL, 97 dB SPL, 93 dB SPL, — — — 89 dB SPL, 85 dB SPL.

This function describes the crossed reflex incorporating the properties of the "crossing neural connections" (Fig. 1). The ipsilateral open loop properties were obtained after subtracting this factor and were described by a different intensity relation for the parameters: Log $t_1 = -0.026x - 1.20$ and $b = 6.50x - 24.9$. x is intensity in dB re reflex threshold.

Decay of closed loop and open loop step response. A linear system has the same frequency transfer function when computed from either positive (onset) or negative (decay) step excitation. Due to the threshold nonlinearity of the middle ear reflex the closed loop resonance peaks are not present in the transfer function calculated from the decay of the closed loop response. On the other hand onset and decay of open loop system responses should be equal. They should also be equal to the decay

of the closed loop responses. Inspection of the time course curves shows that this is not the case (Borg 1972 d). Consequently, onset and decay must be described separately. Furthermore, if consistent resonance peaks are seen in the decay responses they could indicate feed back in addition to the regulation of sound transmission (*e.g.* receptors in the muscles, loops in the brainstem).

Fig. 8 shows the amplitude (left) and phase (right) values of the decay of the closed loop responses to tone bursts of various intensities (upper graphs) and the corresponding open loop responses (lower graph) recorded on the same animal as in Fig. 5 (lower graph). The decay characteristics were intensity dependent but in contrast to the onset the cut-off frequency can be seen for the most part to decrease as a function of stimulus intensity. As a rule, the cut-off frequency for the decay was considerably lower than that for the onset. The amplitude properties of the decay response lacked a main resonance peak at 15—20 Hz, but there were smaller peaks both in the closed loop and the open loop curves. Such peaks were seen in all cases, varying in shape and frequency. It has not been possible to relate them to any biological function.

The decay characteristics were described mathematically by the same methods as the onset properties. It was thus found that the decay could be described by a second-order frequency transfer function $G_o(j\omega) = \frac{K\, e^{-t_1 j\omega}}{(j\omega + a)\ (j\omega + b)}$ where t_1, a and b are intensity dependent parameters: $\log t_1 = -0.029x - 1.73$, $a \cdot b = -0.93x + 31.7$, x is intensity in dB re. reflex threshold (see broken lines of Fig. 7, lower graph). The intensity variation of a and b for the decay (filled triangles) is seen to be considerably less than for the onset. It can furthermore be noticed that the latency (t_1, open triangles) is shorter for the decay response than for the onset response.

Dependence on sound frequency. Transfer functions were calculated for certain animals from the onset of responses to stimulation with 0.5 kHz tone bursts as well as to the standard sound frequency of 2.0 kHz. Since stimulus artefacts often disturbed the time course of ipsilateral responses at high sound level, the recordings from only few animals were available for analysis of properties at 0.5 kHz stimulation. As a rule, the closed loop transfer functions showed more pronounced resonance peaks at stimulation with 0.5 kHz than at 2.0 kHz. The frequency of the resonance peak also varied with stimulus intensity more at 0.5 kHz than at 2.0 kHz. In one typical case the resonance peak varied from 8 to 18 Hz. At 2.0 kHz, resonance peaks were seen only near maximum response (Fig. 4) whereas at 0.5 kHz the peaks were present also well below 50 % of maximal response.

The intensity dependence of the open loop transfer functions at 0.5 kHz stimulus tone was similar to that at 2.0 kHz. However, as compared to the response at 2.0 kHz (a) the range of cut-off frequencies was shifted towards lower frequencies and (b) the phase shift of 180° occurred at a lower frequency. In one typical case the cut off frequency of the crossed total open loop reflex was between 5.6 and 7.5 Hz as determined from responses to 0.5 kHz tone bursts. For responses to 2.0 kHz

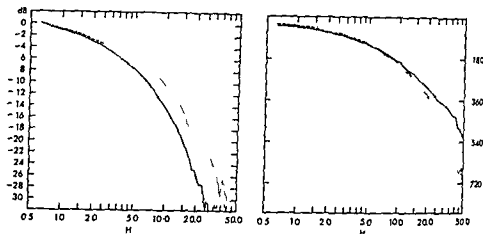


Fig 9 Frequency transfer functions calculated from open loop impulse responses (crossed reflex) ——— 113 dB SPL 105 dB SPL 99 dB SPL

the cut off was between 7 2 and 10 6 at about the same range of response amplitudes in the same animal The high frequency roll off had a slope of 12 dB/octave Phase shift of 180° occurred at 7 5 to 15 0 Hz and 8 4 to 21 Hz respectively for 0 5 and 2 0 kHz tone bursts in the above mentioned typical case The calculated frequency values for 180° phase shift of ipsilateral reflex were between 8 2 and 17 5 Hz and 8 8 and 25 5 Hz for the two sound frequencies respectively This dependence on sound frequency is remarkable and could indicate a frequency selectivity in the reflex arc

Impulse response of the open loop system The open loop transfer function has been studied in man using responses to impulses that were shorter than the system latency (Moller 1962) The results obtained were not consistent with the open loop transfer function determined from the onset of a step function (1450 Hz tone burst) This discrepancy could have been due to the fact that the in stapedius reflex indeed influences the middle ear transmission at 1450 Hz (Borg 1968) Thus the step responses would not represent the true open loop properties The present experimental conditions provided an opportunity to compare the step and impulse responses of the open loop system

The Fourier amplitude and phase values calculated from impulse responses of one typical case at three different sound intensities are shown in Fig 9 (the same animal as in Fig 5 lower graph and in Fig 8) The amplitude transfer functions (left) calculated from impulse responses regularly show less intensity variation than those based on the onset of step response In contrast their cut off frequencies are seen to decrease at increasing sound levels By comparing Fig 9 with Fig 5 and Fig 8 it is seen that the amplitude values have cut off frequencies below those for the onset at least for high intensities but higher than those of the decay However the slope at high frequencies was found to be about the same (12 dB/octave The phase shift (right) approached asymptotes with less slope for higher as compared to lower intensities indicating that the latencies decreased

Thus it is seen that the frequency transfer functions calculated from the impulse responses differed from those of the onset of the step response even during open loop conditions They showed a mixture of onset and decay properties and cannot be used as an easy way to determine the onset open loop properties

B The isolated m stapedius reflex

Closed-loop system The time course of the closed loop responses of the total reflex and of the isolated m stapedius reflex were found to be similar However the isolated m stapedius reflex usually had oscillations of higher amplitude and less damping i e less stability than the total reflex In both conditions the frequency of oscillation was within the same range (18—?? Hz) The transfer function of the isolated m stapedius closed loop system was calculated for one case It showed the expected prominent resonance peaks otherwise it was very similar to the closed loop properties of the total reflex

Open loop system The open loop properties of the isolated m stapedius reflex were not quantitatively analysed The reasons were (a) that the closed loop properties were found to be very similar to those of the total reflex (b) that the steady state stimulus-response curves were very similar (Borg 19?2 c) (c) that the responses in time domain below the threshold for influence on sound transmission (Borg 19?2 d) were similar (Borg 1972 c) Since new results were not expected the requisite technically difficult operations (Borg 1972 c) were not warranted

C The isolated m tensor tympani reflex

Onset step response of closed loop system The m tensor tympani reflex has been found to have negligible influence on the transmission of sound to the cochlea above approximately 2 0 kHz at intensities below about 120 dB SPL (Borg 1972 d) Responses to tone bursts in this range is a good representation of the open loop characteristics of the isolated m tensor tympani response The majority of the recordings of the present study were obtained with 2 0 kHz tone burst stimuli This was near the limit for the influence of the m tensor tympani on sound transmission In fact oscillations in the reflex response were seen in the ipsilateral isolated m tensor tympani responses of two (of eighteen) rabbits to 2 0 kHz tone bursts at 110—115 dB SPL Resonance peaks were observed at 8—10 Hz and 10—12 Hz respectively indicating that a contraction of the m tensor tympani caused attenuation of the stimulus tone

Onset step response of open loop system The open loop transfer functions of the m tensor tympani reflex were calculated from the ipsilateral and the crossed response in animals with the m tensor tympani intact in the stimulated ear and from the crossed response in one animal with both muscles inactivated in the stimulated ear Fig 10 (upper graphs) shows the frequency transfer functions of the onset of the open loop crossed m tensor tympani reflex The intensity dependence of the amplitude and phase of the computed transfer functions are seen to be less prominent than for the total reflex. The slope of the high frequency flank of the amplitude response was about 12 db/octave (in this case and in the 3 cases with an intact ipsilateral m tensor tympani)

The onset open loop properties were approximated by a second-order differential equation in the same way as described for the onset of the total reflex with the corresponding transfer function

$$G(j\omega) = \frac{K e^{-t_1 j\omega}}{(j\omega+a)(j\omega+b)}$$

where t_1 a and b are intensity dependent parameters Log $t_1 = -0\,019x - 1\,20$ a $b = 1\,81x + 0\,6$ x is sound level in dB re tensor tympani reflex threshold The lower left graph of Fig 10 shows the values of the parameters of the crossed m tensor

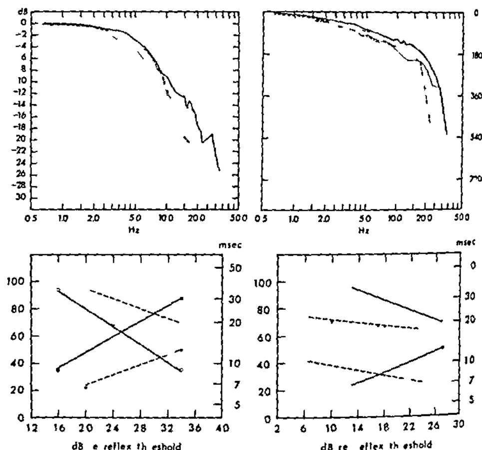


Fig 10 Frequency transfer function calculated from onset of open loop isolated m tensor t m [?]ani responses (upper graphs) Stimulus 2 0 kHz ——— 114 dB SIL 97 % 108 dB SPL 67 104 dB SPL 52 % 100 dB SPL 49 % Lower graphs show intensity dependence of parameters Left onset of m tensor tympani response (triangles) and onset of the total reflex response of the same ear (circles) Intensity is relative to the total reflex threshold Tensor tympani threshold is 7 dB higher than the total reflex threshold Filled symbols show parameters a and b (left ordinate 1/s) open symbols show logarithm of latency (right ordinate) Intensity dependence of parameters of second order transfer function describing total reflex log t₁ = −0 033x−0 91 a, b = 2 85x−8 4 Right onset of m tensor tympani response circles) and decay of m tensor tympani response (triangles another animal) Intensity (x is relative to reflex threshold for each animal

tympani reflex (triangles broken line) and the corresponding crossed total reflex (circles continuous line) as a function of sound intensity relative to threshold of the total reflex All recordings were obtained from the same animal Filled symbols show a and b (equal) and the open symbols represent the logarithmic values of the latency (t_1) It is een that the parameters a and b were smaller for the m tensor tympani reflex than for the total reflex of the same animal when seen in relation to the sound level indicating the m tensor tympani reflex was slower The threshold of the m tensor tympani reflex however was in this case 7 dB higher than that of the total reflex Regarding this difference in excitability the remaining difference in the transfer function is rather small but still definite

The difference between the transfer functions of the ipsilateral and contralateral isolated m. tensor tympani reflex was calculated in two animals in the same way as previously described for the total reflex (*cf.* Fig. 4, bottom graph). At 6.25 Hz the amplitude response was attenuated 1.1 dB more for the crossed than for the ipsilateral reflex and the phase shift was 29° greater. There was no dependence on intensity in contrast to the total reflex (Fig. 4). Above this frequency the difference values showed very poor statistical stability. They could thus not be used for calculations of the ipsilateral m. tensor tympani reflex properties as was done for the total reflex.

Decay of step response. The frequency transfer function for the decay was calculated from the ipsilateral m. tensor tympani reflex system of one animal which showed typical responses in the time-domain. The parameters of the approximated second-order transfer function are shown as triangles in the lower right graph of Fig. 10. The parameters were for the most part independent of stimulus intensity and differed less from the onset properties (continuous line) than was the case for the total reflex (lower graph of Fig. 7). It should be noted though that the decay was calculated from the ipsilateral reflex and the onset from the crossed reflex in Fig. 10, whereas the crossed reflex was used both for decay and onset of the total reflex (Fig. 7).

The decay characteristics are described by a second-order differential equation

$$G(j\omega) = \frac{K e^{-t_1 j\omega}}{(j\omega+a)\,(j\omega+b)}$$

The parameters are functions of intensity (x) in dB re m. tensor tympani reflex threshold. $\log t_1 = -0.006x - 1.62$, $a b = -1.04x + 49.0$.

In general the m. tensor tympani reflex was slower than the total reflex and the dynamic nonlinearities were less prominent. Compensation for the difference in excitability made the difference in parameters values smaller compared to the total reflex but still definite.

Discussion

Use of linear analysis methods

Linear analysis of a dynamic system leads to a description of the properties of the system with linear differential equations (or their Laplace or Fourier transforms). By these equations the behavior of the system can be predicted and the role of the different parts of the system can be evaluated as well as the consequences of changes in system parameters. The validity of the derived equations depends on the linearity of the system. Nonlinear systems can also be analysed with linear methods since they often are approximately linear under certain restricted circumstances. The approximations always lead, however, to limitations in the precision and general validity of the results.

Most systems can be regarded linear in small amplitude ranges. In the present study it was assumed on the basis of earlier experiments (Borg 1971) that each particular step response approximated a step response of *one* linear system.

assumption regarding linearity was taken to be valid for stimuli containing rapid changed in amplitude The validity of the assumption of linearity in the present study is supported by the fact that the same second order transfer function was found to describe accurately the properties at all step response amplitudes by using only intensity dependent parameters The fair agreement between the closed loop resonance frequency (Fig 4) and the frequency where the open loop transfer function has 180° phase shift, also supports the validity of the use of linear analysis methods The mathematical models derived has not yet been tested for stimuli other than positive and negative steps

Since onset and decay properties were found to differ it is not surprising that the impulse response showed properties (Fig 9) intermediate to those of onset and decay The insignificant intensity dependence of impulse transfer functions that was observed was also expected regarding the reversed intensity dependence of onset and decay properties (Fig 7) In man the dynamic properties of onset and decay have been shown to differ (Moller 1962 Dallos 1964 a) Since the impulse response evidently is a function of both onset and decay characteristics it can not be used to approximate the onset open loop transfer properties This failure is unfortunate, since as pointed out by Moller (1962) use of the impulse response would facilitate quantitative analysis of open loop dynamic properties in man

A direct determination of the isolated m stapedius reflex open loop properties was not made in the present study This was regarded unnecessary since the closed loop dynamic properties were found to be similar to those of the total reflex as are the responses below the threshold for attenuation of the sound which shows open loop properties (Borg 1972 c) Furthermore the stimulus response function of the total reflex and of the isolated m stapedius reflex have not been found to differ significantly (Borg 1972 c) Consequently there is good reason to assume that the open loop transfer function of the total reflex closely represents that of the isolated m stapedius reflex as well

The nonlinear dynamic properties of the acoustic middle ear reflex

Two types of nonlinear dynamic behavior were found in the open loop characteristics (a) dependence on intensity and (b) dependence on direction of change in stimulus intensity Similar properties have been observed in acute experiments concerning flexor reflexes (Sherrington 1911) and contractions of external eye muscles (Yamanaka and Bach y Rita 1970) Thus these characteristics may be viewed as inherent to reflex systems In neither of the cited experiments the nonlinearities were quantitatively analysed The amplitude dependent nonlinearity is probably due in part to the organization of the motoneuron pool slow motor units having low thresholds and fast units high thresholds (see *e g* Henneman and Olson 1965 Henneman *et al* 1965 Borg 1971) There is evidence that the central reflex organization of the middle ear muscles is likewise heterogenous containing both slow and fast connection (Borg 1972 f) which could have similar consequences Since the reflex responses at low sound intensity are considerably slower than the motor units (Te

1969 1972) they are probably determined by central mechanisms such as heterogeneity of reflex arc time to reach firing level of neurons and firing rate A decrease of the latency at increase in stimulus intensity has been observed in the middle ear reflex in many experiments (*e g* Lorente de No 1933 1935 McRobert *et al* 1968) Such a decrease can be expected to occur whenever a temporal integration is preceding a threshold, as for example in synapses Furthermore it is well known that rise times of muscle contractions decrease when the frequency of electrical stimulation of the muscle nerve is raised to fusion frequency and even above that frequency (see *e g* Fuchs and Luscher 1971) The actual mechanism underlying the amplitude dependent nonlinearity of the middle ear reflexes is however not known at present

The decay properties were found to be slower than those of the onset but surprisingly they had a shorter latency This might indicate that the muscles have no active resting tonus and that it takes some time during contraction to reach threshold for firing of motoneurons and also for influence on the acoustic properties of the ear The decay properties would thus give a better estimate of the transport time than the onset properties The lack of active tonus is supported by the fact that experiments designed to test the presence of myotatic reflex mechanisms in the m stapedius have shown negative results (Wigand and Brauer 1964) The reversed and less prominent intensity dependence of the decay parameters as compared to those of the onset as was found in the present study can be explained as an effect of after discharge After discharge is common in polysynaptic reflexes (Sherrington 1911) especially at high stimulus intensities as has also been observed in the EMG of the stapedius muscle (see *e g* Wersäll 1958 Borg 1972 a)

The difference in the speed of onset and decay that was seen foremost in the total reflex and in the isolated m stapedius reflex response can be regarded as an example of unidirectional rate sensitivity (URS see Clynes 1961 1962) URS is a nonlinear property of a system implying sensitivity for speed of the stimulus only in one direction. Model systems with a combination of proportional and URS properties have been found to respond to square wave stimulation in a way very similar to the middle ear reflex (Clynes 1962 *cf* Borg 1972 d) In acute experiments in the cat low threshold tonic and high threshold phasic motor units have been recorded in the middle ear muscles (Okamoto *et al* 1954) as in other skeletal muscles (Granit *et al* 1956) It is thus possible that also the direction dependent nonlinearity of the acoustic middle ear muscle reflexes is due to the organization of the motoneuron pool

Functional significance of the dynamic properties

The responses of the middle ear reflexes can be recorded both in animals and in man with identical methods and quantitative comparisons can be made with regard to the fundamental properties of their function (Borg 1972 c d) On this fundament the analysis can be widened in man by correlating the basic physiologic properties to psychoacoustic phenomena and in animals by relating them *e g* to the functio of other parts of the efferent auditory system or to sound induced damage to the

The middle ear reflex is a biomechanical system with very small moving mass. It could therefore be expected to be faster than most other mechanical systems in the body. This is not, however, the case. The natural frequency of the m. stapedius reflex in man, for example, is 5 Hz (Møller 1962), whereas in the monosynaptic proprioceptive feed back loop responsible for finger tremor it is 8—12 Hz (Lippold 1970), and in the visual feed back loop giving finger tremor it is about 9 Hz (Merton et al. 1967). These latter systems have greater mass, longer nerve tracts and the last mentioned may well have more synapses than the m. stapedius reflex. Thus there is a discrepancy between the actual speed of contraction of the middle ear muscles and the much greater speed that could be expected. This difference suggests that the actual dynamic properties of the middle ear reflexes represent the optimal compromise to several functional demands, e.g. high gain, stability and adaptation to the dynamic properties of the regulated system. For example, some psychoacoustic phenomena have remarkably slow temporal characteristics. The integration time in the system for loudness perception in man is approximately equal to that of the m. stapedius reflex, 200 ms (Zwislocki 1969, Djupesland and Zwislocki 1971) and the threshold for detection of amplitude modulation is lowest at a modulation frequency of 3 to 4 Hz (Zwicker and Feldtkeller 1967).

It can also be observed in this connection that the dynamic properties of olivocochlear feed back regulation in the cat are similar (Fex 1962) to those of the middle ear reflexes in the rabbit.

The amplitude dependent dynamic nonlinearity is also interesting when regarded as an example of adaptation of the properties of the regulator to those of the regulated system. Although the dynamic properties of the development of sound induced fatigue and damage to the cochlea are not known in detail, it is clear that damage develops faster the higher the sound level (for review see Kryter 1970), which thus is compatible with a faster attenuation of high level sound in the middle ear. Furthermore, both amplitude dependent and direction dependent non linearity have been shown to improve the stability of a feed back loop simulated by a digital computer (Borg 1972 e).

Thus the temporal chracteristics of the middle ear reflexes seem to be related to some important properties of the auditory system in a quantitatively systemic manner.

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Enhancement by Indomethacin of Cold Induced Hypersecretion of Noradrenaline in the Rat in vivo - by Suppression of PGE Mediated Feed back Control?

By

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Abstract

STJÄRNE L *Enhancement by indomethacin of cold induced hypersecretion of noradrenaline in the rat in vivo — by suppression of PGE mediated feed back control?* Acta physiol scand 1972 *86* 388—397

While indomethacin treatment did not alter the urinary excretion of noradrenaline in the rat at room temperature it consistently and significantly augmented the urinary hyperexcretion of noradrenaline induced by exposure to cold the excretion of adrenaline remained initially unchanged The effect of indomethacin increased progressively with time during the experimental period of about one week The results imply that indomethacin which is known to be able to block the formation of PGE *in vivo* as well as *in vitro* augments the secretion of noradrenaline from sympathetic nerves during sympathetic hyperactivity Since the amount of noradrenaline secreted as a result of nerve stimulation is raised when PGE formation is depressed in isolated tissues *in vitro* the present result may imply that the sympathetic hypersecretion induced by indomethacin was secondary to suppression of PGE formation

Recent evidence from studies of sympathetic neurotransmitter secretion carried out in different laboratories has led to the novel concept in physiological cybernetics that the amount of transmitter secreted from sympathetic nerves may be controlled by some kind of local feed back mechanism

One line of experiments has led to the implication that endogenous prostaglandins may be the chemical mediators of such local control This work was inspired by observations originally made by Brundin (1968) in studies of the inhibitory effects of exogenous prostaglandin E_1 (PGE_1) on the contraction of the smooth muscle of the isthmic parts of the Fallopian tube induced by sympathetic nerve stimulation In subsequent work with the isolated perfused cat spleen Hedqvist and Brundin (1969) presented evidence that part of the inhibitory effect of exogenous PGE_1 on sympathetic neuroeffector transmission may be exerted prejunctionally by depression of the amount of noradrenaline (NA) secreted from the sympathetic nerves on arrival of the propagated nerve impulse Hedqvist showed that the inhibitory effect

of prostaglandin E (PGE) on NA secretion is even more marked than that of PGE_1 (Hedqvist 1969 a) He further found (Hedqvist 1969 b) that the powerful potentiating effect of phenoxybenzamine (PBA) on the nerve stimulation induced secretion of NA (Brown and Gillespie 1957) could be counteracted by exogenous PGE_2 at concentrations which might well be attained locally as a result of local release of endogenous PGE triggered by events induced by sympathetic nerve activity He suggested (1969 b) that at least part of the potentiating effect of PBA on NA secretion might be due to a PBA induced block of local release of endogenous PGE He further proposed that the secretion of sympathetic neurotransmitter in general may normally be controlled by endogenous PGE locally produced and released as a result of sympathetic nerve activity (Hedqvist 1969 a) The observations concerning the effects of exogenous prostaglandins originally made in the isolated cat spleen have by now been amply confirmed in work with several isolated tissues from various species (for refs see Hedqvist 1970 Swedin 1971 Wennmalm 1971 Hedqvist and Euler 1972) An important further development was the demonstration that pharmacological blocking of the local formation of PGE leads to considerable increase in the outflow of NA induced by stimulation of the sympathetic nerves of the isolated rabbit heart (Samuelsson and Wennmalm 1971) Subsequently similar observations have also been made in the vas deferens from several species (Swedin 1971 Hedqvist and Euler 1972) and in the cat spleen (Hedqvist Stjärne and Wennmalm 1971)

By a different approach Häggendal (1970) was led to the conclusion that the secretion of sympathetic neurotransmitter may be controlled by a local feed back mechanism He found that the potentiating effect of PBA on NA secretion from sympathetic nerves could not be explained exclusively on the basis of improved tissue perfusion and inhibition of reuptake into the nerves of NA secreted Since part of the effect of PBA might be secondary to blocking of α receptor mediated smooth muscle contraction he concluded that alteration of the mechanical state of effector system may somehow be fed back to the sympathetic motor nerves and restrict further secretion of transmitter

Thus results from different lines of experimentation suggest that the very process of secretion of neurotransmitter on arrival of the propagated nerve impulse may be subject to physiological control The present study was made to test the possibility that endogenous PGE is the chemical link mediating such feed back control by studying how suppression of local PGE formation in the rat *in vivo* affects the secretion of NA from sympathetic nerves The pharmacological tool used was indomethacin (Indomee® Merck Sharp & Dohme) which has recently been shown to block the formation and/or release of PGE in isolated tissues (Ferreira Moncada and Vane 1970 Vane 1970) as well as *in vivo* (Hamberger and Samuelsson 1972) The secretion of NA from the nerves was monitored by determining the urinary excretion of NA under conditions of low sympathetic nerve activity at room temperature as well as during periods of increased load on the sympathetic nervous system induced by intermittent exposure to cold The present work is a detailed

presentation and extension of a preliminary paper (Stjarne 1971), in which it was reported that indomethacin 5 mg/kg/day consistently and significantly raises the urinary hyperexcretion of NA during cold exposure as compared to the level obtained in controls receiving vehicle

Material and methods

60 female Sprague Dawley rats with an initial body weight of about 190 g were used for the experiments The rats were kept in metabolic cages occasionally one but usually two rats in each cage Food and water were given *ad libitum* Urine was collected in 24 h fractions in bottles containing 10 ml of N HCl Small plugs of glass wool in the plastic funnels of the cages prevented contamination of the urine collected with faeces the funnels were rinsed daily with a 10 ml volume of 0 1 N HCl which was added to the urine collected

After an initial period of about one week for adjustment to the metabolic cages the rats were given a single daily dose of indomethacin (Indomee® MSD 0 5—15 mg/kg of indomethacin in powder form mixed with an equal amount (by weight) of lactose and suspended in 1% carboxy methyl cellulose) in a 0 5 ml volume orally by gavage each day at about 5 p m Control rats received the same amount of vesicle

In some experiments the rats were kept at room temperature throughout In others they were transferred to the cold room (+2 to +4 C) for 15 h each 24 h period after 2 initial days at room temperature

The daily urinary excretion of free catecholamines (CA) was determined fluorimetrically after purification on alumina (Euler and Lishajko 1961) A certain loss of CA during the collection period is unavoidable and difficult to estimate the average overall recovery of NA slowly sprinkled on the metal wire net floor of the cages in a 10 ml volume of fresh urine and processed over alumina according to the routine procedure was 57 3±1 4 % The values given for urinary excretion of A and NA are uncorrected

The possible effects of indomethacin on the fate of circulating catecholamines i e on uptake in the tissues and on enzymatic degradation were tested by determination of the proportion of subcutaneously injected ^{3}H NA (0 5 μCi in 0 5 ml 0 9 % NaCl New England Nuclear Corp Specific activity around 5 Ci/mmol) which was excreted in the urine during the following 15 h as free intact amine

In the groups exposed to intermittent cold 12 control rats and 10 rats treated with indomethacin 5 mg/kg/day were sacrificed on the 7th—8th day for determination of the effect of indomethacin on tissue content of CA The rats were killed by decapitation (in half of the cases after brief ether anesthesia and in the remainder after a blow on the head no differences were observed between the two groups) Brain heart spleen and adrenals were rapidly dissected out and homogenized in 5—10 vol of 10 % tricloroacetic acid After purification on alumina the CA were determined fluorimetrically Recovery was monitored by addition of tracer amount of tritium labelled NA the values given for organ content of NA are corrected for losses

Results

The dose range of indomethacin used was 0 5—5 mg/kg/day (in a few cases the dose was further raised to 10 and 15 mg/kg/day) orally by gavage in a single dose at 5 p m No gross behavioural effects were noted during the first 3—5 days at a 5 dose level However after this stage the spontaneous motor activity of the rats given 5 mg/kg/day gradually declined they started to appear flaccid on handling and they did not eat as well as previously These symptoms were most distinct in the rats exposed to intermittent cold but also appeared in those kept at room temperature There was marked variation in the intensity of the symptoms between different experimental groups However starting on the 4th or 5th day of cold exposure of rats given the 5 mg/kg/day dose the volume of faeces declined and there was a significant

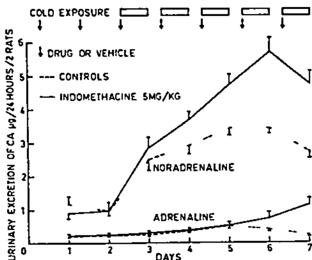


Fig 1 Effect of indomethacin 5 mg/kg/da on the urinary excretion of NA and A in rats kept at room temperature for 2 days and subsequently exposed to +2 — to +4 C for 15 h each 24 h period Comparison to controls receiving vehicle Drug group 18 rats control group 18 rats 2 rats were kept in each cage Mean ± S E

fall in urinary volume ($p < 0.01$) At this stage 4 out of 36 rats given this dose of indomethacin and exposed to cold died while no control rats nor any of those given 0.5–2 mg/kg/day were lost during the 5 days of intermittent cold exposure

After subcutaneous injection of ^{3}H l NA 5.2 ± 0.4 % of the injected dose was excreted in the urine as free intact amine No differences were found in this respect between control rats and those receiving indomethacin 0 5 or 5 mg/kg/day neither at room temperature nor in the cold

The average urinary excretion of NA and A of rats kept at room temperature two in each metabolic cage was 2 78 µg/kg/24 h and 0 6 µg/kg/24 h respectively Under these conditions no effect was seen of indomethacin 5 mg/kg/day for 2 consecutive days nor did this dose of the drug significantly alter the urinary excretion of catecholamines of single rats kept at room temperature and given indomethacin 5 mg/kg/day for 5 days

During the first day of cold exposure the urinary excretion of NA in the control rats rose steeply while the A level remained unchanged (Fig 1) On continued intermittent exposure to cold there was a gradual rise in the excretion of both amines to reach maximum level on the third day After that the excretion of NA as well as of A declined on the fifth day the NA and A levels had returned to those found during the first day of exposure to cold

As mentioned above indomethacin 5 mg/kg/day did not significantly alter the urinary excretion of either NA or A at room temperature However on exposure to cold the drug consistently caused a further rise in the excretion of NA but initially not of A above the level of the cold exposed control rats (Fig 1) This effect of indomethacin treatment became apparent already during the first day of exposure to cold ($p < 0.05$) but it became gradually more marked with time (Fig 2) While on the 4th to 5th day of exposure to cold the A levels of control rats declined th

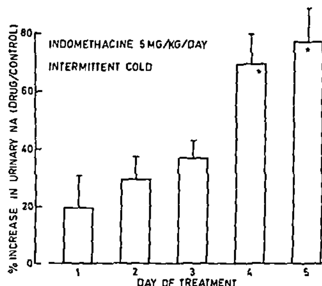


Fig 2 Time course of indomethacin effect on urinary excretion of NA in rats during intermittent exposure to cold expressed as per cent increase in drug group (n = 18) compared to control group (n = 18) Mean ±S E

of the indomethacin treated rats continued to rise (Fig 1) There was a considerable individual variation in this late A response but on the 5th day the difference to the controls was very marked and statistically significant ($p<0.001$)

In order to test the dose response relationship of the effect of indomethacin treatment on the urinary excretion of NA and A a separate experiment was performed in which a group of 24 rats was divided into 12 pairs the rats were exposed to intermittent cold and given vehicle or indomethacin 0 5 2 or 5 mg/kg/day respectively The results are shown in Fig 3 which clearly demonstrates the absence of a regular dose response relationship If the data for the whole five day period are pooled the results imply that indomethacin treatment at all 3 dose levels caused a highly significant ($p<0.001$) increase in the urinary excretion of NA as compared to that of the control rats Pooling the data from the first 3 days of treatment shows that indomethacin at 0 5 and 2 mg/kg/day caused an about equal and statistically significant ($p<0.01$) increase in the urinary excretion of NA while the effect of 5 mg/kg/day was markedly higher ($p<0.001$ when compared to controls) the difference between the effect of the higher dose and that of the two lower doses was not statistically significant however On pooling the data from the 4th and 5th day of treatment when the urinary excretion of A in the 5 mg/kg/day group was rising (Fig 1) it was found that the effect of indomethacin 0 5 and 2 mg/kg/day was possibly declining however these dose levels still caused a significant ($p<0.01$) rise in urinary NA when compared to control rats During this period indomethacin 5 mg/kg/day caused a much more marked elevation of urinary NA than at the two lower dose levels ($p<0.001$)

At this stage as mentioned above altogether 4 out of the 36 rats given the 5 mg/kg/day dose and exposed to intermittent cold died this is the reason why the

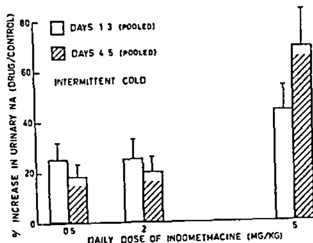


Fig 3 Dose response relationship of indomethacin effect on urinary excretion of NA in rats during intermittent exposure to cold expressed as per cent increase in drug group compared to control group receiving vehicle 6 rats in control group and in each dose group ? rats in each cage Pooled data from days 1—3 (n = 9 in each group) and days 4—5 (n = 6)

experimental period in the present study was limited to 5 days in the cold The rats exposed to cold and given indomethacin 5 mg/kg/day for this time period weighed less than control rats ($p<0.001$) no such reduction was seen in rats given 0 5 or 2 mg/kg/day of indomethacin Peritoneal exsudate was found in the 4 rats which died spontaneously The stomach of all of the indomethacin treated rats was markedly distended although to a variable extent There was no difference in weight of heart or brain but spleen ($p<0.01$) and adrenals ($p<0.05$) were markedly enlarged in the indomethacin treated rats

The NA content (Table I) of heart ($p<0.001$) and adrenals ($p<0.01$) was reduced when compared to that of controls while the NA level of brain was somewhat elevated ($p<0.05$) NA in spleen and A in adrenals of the indomethacin treated rats did not differ from control values

TABLE I C t cholamine content of heart spleen brain and adrenals Mean ± S E

	Heart NA µg/g	Spleen NA µg 2 spleens	Bra n NA µg/g	Adrenals A µg 4 glands	NA µg 4 glands
Controls	1 32 ± 0 07 n = 6	1 23 ± 0 06 n = 6	0 64 ± 0 02 n 6	54 9 ± 3 3 n = 6	9 1 ± 0 6 n = 6
Indom thacin treated	0 92 ± 0 08 n = 5	1 13 ± 0 07 n = 5	0 71 ± 0 00 n = 5	59 4 ± 4 7 n = 5	5 6 ± 0 7 n = 5
D ffe ence	p < 0 001	—	p 0 05	—	p < 0 01

[1] Dose 5 mg/kg/day for 7—8 days (first two days at room t mperatu e after that 15 h in the cold each 24 h period)

Discussion

The aim of the experiments was to study the effects of treatment of rats with an inhibitor of PGE formation indomethacin on the secretion of sympathetic neurotransmitter as reflected in the urinary excretion of NA, under conditions of low sympathetic nerve activity as well as during sympathetic hyperactivity induced by exposure to cold

The values obtained for urinary excretion of NA and A both at room temperature and in the cold agree well with those reported by Leduc (1961) the comparison is somewhat complicated by the fact that he used single male rats and continuous cold exposure while in the present study paired female rats were exposed to intermittent cold

There is general agreement that the major proportion of the NA excreted in urine under most experimental condition (Euler 1956) including those prevailing when urine is collected from rats at room temperature or during exposure to cold (Leduc 1961) is derived from sympathetic nerves Only during severe stress does NA derived from adrenal medulla significantly contribute to the total urinary excretion of NA (Leduc 1961)

Since indomethacin treatment did not alter the relative urinary excretion of [^{3}H]NA as free intact amine the present results give good reason to assume that the indomethacin induced increase in the urinary excretion of NA above the level observed in controls mainly reflects increased secretion of NA from sympathetic nerves However during the 4th and 5th day of cold exposure of rats given the 5 mg/kg/day dose there were signs of increased adrenomedullary secretory activity a rise in urinary A and a fall in NA content of adrenal medulla During this period an increased proportion of the NA excreted in urine was probably derived from adrenal medulla With this exception there is reason to assume that variations in urinary NA gave a valid reflection of variations in secretion of NA from sympathetic nerves

The results obtained confirm and extend the observations previously reported (Stjärne 1971) that indomethacin 5 mg/kg/day significantly enhances the urinary hyperexcretion of NA in rats exposed to intermittent cold

At room temperature there were no consistent effects of indomethacin at this dosage on urinary excretion of NA under the experimental conditions These results are apparently at variance with those of Junstad and Wennmalm (1972) who report that single daily subcutaneous injections of 0.5—2 mg/kg/day of an indomethacin preparation not containing lactose and dissolved in phosphate buffer raises the urinary excretion of NA of rats kept at room temperature However in this study indomethacin further enhanced the urinary excretion of NA already considerably elevated as a result of injection of the buffer alone the discrepancy may thus be apparent only and the results may be compatible with the present finding that the enhancing effect of indomethacin treatment on urinary excretion of NA is much less readily demonstrable during conditions of low sympathetic nerve activity than during sympathetic hyperactivity

The effect of the 5 mg/kg/day dose increased progressively with time From the 4th day in the cold the general condition of these rats began to deteriorate and the rising excretion of A indicates increasing adrenomedullary discharge presumably of NA as well as of A However during day 6 (day 4 in the cold) the very high level of urinary NA in the presence of an only moderate rise in A (*cf* Fig 1) indicates that adrenomedullary NA probably contributed only to a minor extent to the overall rise in NA excretion It is thus likely that the major part of the urinary NA at this time was derived from sympathetic nerves The present results give no clue to decide whether this was due to an increased possibly reflexly induced firing frequency in these nerves or to an increase in the amount of sympathetic transmitter secreted per nerve impulse

It is interesting that a dose as low as 0 5 mg/kg/day had a highly significant elevating effect on the urinary hyperexcretion of NA in the cold since this is the highest dose tolerated in the rat over a prolonged period of treatment this dose has the full anti inflammatory effect typical of indomethacin (personal communication Merck Sharp & Dohme Research Lab) Raising the dose to 2 mg/kg/day did not further enhance the effect on urinary NA while 5 mg/kg/day already during the first 3 days in the cold had a higher effect It is difficult to comment on the tendency to a gradual decline in the response to 0 5—2 mg/kg/day doses contrasting to the progressive rise in that to 5 mg/kg/day Occasional further increase of the dose to 10 or 15 mg/kg/day did not further enhance the effect seen with 5 mg/kg/day (Stjarne 1971)

As mentioned in the introduction the present experiments were performed on the working hypothesis that the secretion of sympathetic neurotransmitter is normally restricted by a PGE mediated braking mechanism (Hedqvist 1969 a) If the hypothesis is correct suppression of PGE formation and/or release should at any given sympathetic firing frequency enhance the secretion of NA which would lead to a rise in urinary excretion of NA

The present results are compatible with such a mechanism for the observed effects of indomethacin However it is not known whether this drug at the dose level used actually depresses PGE release in the rat The dose required to produce 98 % inhibition of PGE release in the guinea pig is much higher 100 mg/kg/day (Hamberg and Samuelsson 1972) However the guinea pig is known to tolerate much higher dosage of indomethacin than the rat (personal communication Merck Sharp & Dohme research Lab) in the rat the dose range 0 5—5 mg/kg/day used in the present study may be sufficient to depress PGE release to a significant extent This possibility is supported by the time course of the indomethacin induced inhibition of PGE release in the guinea pig it became progressively more complete with time over the first 3 days of treatment (Hamberg and Samuelsson 1972) in a manner reminding of the progessive increase with time of the effect of the 5 mg/kg/day dose on urinary NA seen in the present study (Fig 2)

Even if indomethacin did depress PGE formation in the present experiments the question remains whether this was the immediate cause of the increased sec

of NA from sympathetic nerves reflected in the observed urinary hyperexcretion of NA in the cold This seems likely since blocking of PGE formation in isolated tissues augments the secretion of NA on electrical stimulation of sympathetic nerves (Samuelsson and Wennmalm 1971 Swedin 1971, Hedqvist Stjarne and Wennmalm 1971 Hedqvist and Euler 1972) However as discussed above the present observations do not exclude the possibility that indomethacin caused further elevation of the secretion of NA on exposure to cold by further raising the firing frequency in sympathetic nerves above the level of untreated cold exposed controls There is in fact reason to assume that reflex sympathetic activity was enhanced in the cold exposed rats given the 5 mg/kg/day dose but only during the 4th and 5th day in the cold when their general condition clearly deteriorated At lower doses of indomethacin and during the first 3 days of cold exposure even in the rats receiving 5 mg/kg/day there is no reason to suspect that the drug treatment as such altered the reflex activity in the sympathetic nerves Thus in view of the evidence from isolated tissues suppression of a PGE mediated braking mechanism normally restricting NA secretion may well be involved in the hyperexcretion of NA induced by indomethacin in the present experiments

In that case the question arises why no effects of indomethacin on NA excretion were demonstrable during low sympathetic activity at room temperature while they occurred with complete regularity during high sympathetic activity in the cold One possible explanation is based on the observation of Green and Samuelsson (1971) that exposure to cold raises the release of PGE in the rat as reflected in urinary excretion of metabolites 2—2 5 fold (incidentally to about the same extent as the normally occurring rise in urinary excretion of NA the rise in PGE might well be the result of increased load on the hypothetical PGE mediated feed back system controlling sympathetic neurotransmitter secretion) since exposure to cold gives a high level of PGE it provides a more suitable background for studies of inhibition of PGE formation and/or release

Thus to summarize treatment with the PGE synthesis inhibitor indomethacin enhances the secretion of NA from sympathetic nerves in rats exposed to cold but not in rats kept at room temperature The effect is compatible with the interpretation that suppression of PGE release disinhibits secretion of NA from sympathetic nerves *The results may thus represent the first demonstration in vivo* that sympathetic neurotransmitter secretion at least during sympathetic hyperactivity is normally restricted by a PGE mediated feed back control system

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Acta physiol scand 1972 86 398—409

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Depression of ^{3}H Glucose Uptake into Rabbit Polymorphonuclear Leukocytes by Glucocorticoids in Concentrations Partly Saturating the Specific Glucocorticoid Uptake Evidence for a Glucocorticoid Receptor

By

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Abstract

SIMONSSON B *Depression of ^{3}H glucose uptake into rabbit polymorphonuclear leukocytes by glucocorticoids in concentrations partly saturating the specific glucocorticoid uptake Evidence for a glucocorticoid receptor* Acta physiol scand 1972 *86* 398—409

Rabbit polymorphonuclear leukocytes have a specific glucocorticoid uptake *in vitro* half saturated by cortisol at a probably physiological concentration (about 3×10^{-9} M) These cells *in vitro* accumulate ^{3}H D-glucose and ^{3}H 2 deoxy D glucose possibly by a common uptake step Cortisol dexamethasone 9αF prednisolone prednisolone triamcinolone and triamcinolone acetonide all in the low concentrations sufficient to partly saturate the specific glucocorticoid uptake depress the uptake of ^{3}H glucose by these cells Corticosterone acted as a partial agonist 11 epicortisol tetrahydrocortisone and progesterone which have no glucocorticoid activity had a very low activity on ^{3}H D glucose uptake For 11 epicortisol and tetrahydrocortisone this corresponded to a low affinity to the specific glucocorticoid uptake process while progesterone had a relatively high affinity Progesterone in high concentrations was shown to block the depressing effect on ^{3}H D-glucose uptake by cortisol probably by keeping it away from the specific glucocorticoid uptake process Rabbit polymorphonuclear leukocytes thus seem to have a specific glucocorticoid receptor

In a previous paper (Simonsson 1972) it was shown that rabbit PMN[1] *in vitro* have a specific cortisol uptake probably corresponding to an interaction with a glucocorticoid receptor The uptake was half saturated at about 1 ng cortisol per ml medium (3×10^{-9} M) which probably is a physiological concentration for the rabbit

[1] *Abbreviations* PMN stands for pseudoeosinophilic polymorphonuclear leukocyte(s) cpm is counts per minute Corticosterone denotes 11β 21 dihydroxy-4 pregnene 3 20-dione cortisol 11β 17 21 trihydroxy-4 pregnene 3 20-dione cortisone 17 21-dihydroxy 4 pregnene 3 11 20-trione dexamethasone 9α fluoro-11β 17 21 trihydroxy 16α methyl 1 4 pregnadiene 3 20-dione 11-epicortisol 11α 17 21 trihydroxy-4 pregnene 3 20-dione prednisolone 11β 17 21 trihydroxy 1 4 pregnadiene 3 20-dione progesterone 4 pregnene 3 20-dione tetrahydrocortisone 3 17 21 trihydroxy 5β pregnane 11 20-dione triamcinolone 9α fluoro-11β 16α 17α 21 tetrahydroxy 1 4 pregnadiene 3 20-dione triamcinolone acetonide 9α fluoro-11β 21-dihydroxy 16α 17α isopropylidenedioxy 1 4 pregnadiene 3 20-dione

(Ganjam *et al* 1970) In a variety of steroids the affinity to the cortisol uptake process roughly increased with increasing *in vivo* glucocorticoid activity (in rats and mice)

Glucocorticoids are known to cause a reduction in glucose utilization or in uptake of labelled glucose by some tissues *in vitro* (Rauch *et al* 1961 Munck 1962 Fain 1964 Matsui and Plager 1969 Rosen *et al* 1970 Gray *et al* 1971) The present work was undertaken to find out whether glucocorticoids in the low concentrations sufficient to partly saturate the glucocorticoid uptake had any effect on the ^{3}H glucose uptake by the PMN This was the case and thus strengthens the evidence for a specific glucocorticoid receptor in these cells

Material and methods

Compounds 6 ^{3}H D-glucose (8 45 Ci/mM) generally labelled H 2-deoxy D glucose (9 Ci/mM) and ^{14}C-inulin-carboxyl (4 1 mCi/g) were from NEN Chemicals Boston Mass The radiochemical purity of the sugars was over 97 % at delivery and they were used within —3 months D-Glucose was obtained from J T Baker Chem Co Philipsburg N J The sources of the steroids and glycogen were the same as in Simonsson 1972 The steroids were in most cases delivered with certificates of analysis The purity was also checked by chromatography on thin layers of silica gel in benzene-ethanol (8 1)

Preparation of polymorphonuclear leukocytes

Details of the procedure have been published in a previous work (Simonsson 1971

Intact male albino rabbits weighing 2.5—4 kg were injected intraperitoneally with warm (33 C) pyrogen free 0 9 % (w/v) NaCl containing 0 1 % glycogen 4 h later 110 ml warm 0 9 % NaCl containing 0 5 % (w/v) Mg EDTA was injected The abdomen was gently kneaded and then the fluid drained by gravity into a collecting flask Smears showed that 95—99 % of the white cells were PMN The volume of erythrocytes varied around 3 of the total cell volume in the exudate The rabbits were injected once a week and they were primed with at least 3 injections before they produced exudates rich enough in leukocytes to be used in the experiments

Measurement of the uptake of ^{3}H glucose and ^{3}H deoxy glucose

The method has many similarities with the measurement of ^{3}H-cortisol uptake in these cells The details of the procedure have been published earlier (Simonsson 1971)

After the peritoneal exudate had been filtered through 3 layers of gauze to remove any clumped cells and fibrin it was centrifuged and washed twice in ice-cold Eagle's medium (Eagle 1955) without phenol red but with doubled concentration of amino acids and finally resuspended in the same cold medium to a concentration of 2—3 × 10 cells per ml Cells were pooled from 2—4 animals at each experiment Two ml samples were then incubated at least in triplicate without steroid or with different concentrations of a steroid present (added in ethanol) If not stated otherwise the cells were incubated at 37 C for 110 min ^{3}H-glucose or ^{3}H-deoxy glucose was added to the samples 40 min prior to the end of the incubation The final concentrations of the labelled hexoses were 17 ng/ml (= 0 8 Ci/ml) and 9 ng/ml (= 0 5 μCi/ml) respectively This should be compared with the concentration of unlabelled glucose 1 mg/ml in the medium The incubation was terminated by centrifugation at 300 × *g* at 0 C for 4 min To reduce the amount of tritium in the extracellular medium each sample was then washed for 5 min at 0 C by resuspending in 2 ml phosphate buffer and finally spun down in the cold at 670 × *g* for 5 min To determine the extracellular volume in the cell pellet ^{14}C-inulin was added immediately prior to the final centrifugation The pellets were dried over night at 100 C and then weighed The pellets and the supernatants were then digested by Soluene® and the radioactivity was analyzed by liquid scintillation. The extracellular cpm tritium trapped in the cell pellet was calculated from the ^{14}C inulin data and subtracted from the total cpm tritium in the pellet The cellular uptake was then expressed as cpm × mg dry weight^{-1}/cpm × μl medium^{-1} Corrections for differences in quenching were made

TABLE I The effect of doubled concentration of non radioactive glucose on the uptake of ³H glucose and ³H deoxy glucose The cells were incubated as in Methods with 1 or 2 mg glucose per ml medium for 110 min (12/71 1/72) or 145 min (33/71) ³H glucose or ³H deoxy glucose was present *only during the last 40 min of the incubation* There were 3–4 samples in each group

Expt	Label	Glucose (mg/ml)	Uptake±S E (cpm/mg / cpm/µl)	Reduction in uptake (%)
12/71	³H glucose (17 ng/ml)	1	2 7±0 03	
		2	1 0±0 1	63
1/72	³H glucose (17 ng/ml)	1	6 0±0 2	
		2	2 3±0 1	62
33 71	³H Deoxyglucose (9 ng/ml)	1	5 4±0 04	
		2	3 1±0 03	42

Results

Rabbit PMN accumulate ³H glucose and the probably non utilizable ³H-deoxy glucose *in vitro* Table I shows that the uptake of both labelled hexoses is suppressed by non radioactive glucose in agreement with a common uptake step for these hexoses The time courses for the uptakes are shown in Fig 1 In these experiments the labelled hexoses were present from the start The amount of ³H glucose taken up is maximal at 110 min and after that time the curve begins to decline A corresponding decrease is not seen in the ³H deoxy glucose curve For both hexoses the amount of radioactivity taken up during a 40 min incubation is however reduced with increasing pre incubation time in medium without labelled hexoses (Fig 2) Since the same effect is seen with ³H glucose as with ³H deoxy glucose it probably is due to an increasing drop in the ability of the cells to accumulate the sugar Very probably this increasing failure is responsible for the flattening off fo ³H deoxy glucose and the downward course at longer time for ³H glucose seen in Fig 1

Fig 1 and 2 also show the effect of cortisol (1000 ng/ml) on the uptake of the labelled hexoses Both uptakes are decreased by the steroid The effect becomes visible a little earlier on the ³H glucose uptake than on the ³H deoxy glucose uptake (within 110 and 150 min in Fig 1 and within 70 and 110 min in Fig 2 respectively) An incubation time of 110 min with the ³H glucose present only during the last 40 min (as in Fig 2) was chosen as a suitable compromise between sensitivity to cortisol inhibition and the decrease in accumulation for the experiments following below

The log dose response curve for the cortisol inhibition of ³H glucose uptake by PMN is shown in Fig 3 For comparison the log dose response curve for the cortisol depression of the specific (saturable) ³H cortisol uptake in rabbit PMN (from Simonsson 1972) is inserted in Fig 3 Both curves are obviously steepest in the

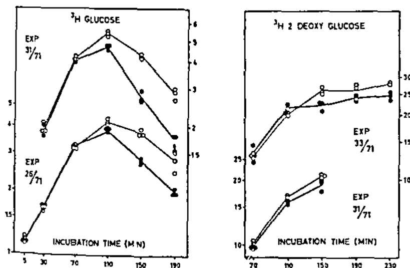


Fig 1 The time courses for the uptake of ^{3}H glucose and ^{3}H-deoxy glucose in the absence (○) or presence (●) of cortisol (1000 ng/ml) The cells were incubated as described in Methods but with the labelled sugar present already from the start The curves connect the arithmetical means of each group The ordinates indicate cpm × mg $^{-1}$/cpm × µl $^{-1}$ the right ones for the upper curves and the left ones for the lower curves

range 0 3—10 ng cortisol per ml medium In Fig 3 the inhibition of ^{3}H glucose uptake by 1000 ng cortisol per ml was not different from that by 10 ng/ml On an average (13 expts) the inhibition of ^{3}H glucose uptake was 25 ± 2 % at 10 ng/ml and 27 ± 2 % at 1000 ng/ml The curve thus has levelled off at 1000 ng cortisol per ml

Log dose response curves were then determined for a number of steroids A cortisol dose response curve was run as a control in each experiment ED $_{0(g)}$ is the concentration of a steroid that gives 50 % of the inhibition produced by 1000 ng cortisol per ml medium in the same experiment The inhibition brought about by the more potent glucocorticoids at 1000 ng/ml was not significantly different from that of cortisol (Table II)

The variations in ED $_{0(1\ 0)}$ for cortisol are shown in Table III The mean value is 2 0 ng/ml (6 × 10^{-9} M) This concentration is remarkably close to the concentration (around 1 ng/ml) that gives half saturation of the specific glucocorticoid uptake in these cells (Simonsson 1972)

Based on the ED$_{50}$s the relative potency (versus cortisol) to inhibit ^{3}H glucose uptake was calculated for a number of steroids (Fig 4) In Fig 4 is also included the relative affinity (cortisol = 1 0) of the steroids to the specific glucocorticoid uptake process in the PMN (from Simonsson 1972) Each symbol in Fig 4 represents 1 exp Progesterone however was run at 300 and 1000 ng/ml in 8 ^{3}H glucose expts For both concentrations of progesterone in each exp the depression of the ^{3}H glucose uptake was compared with that caused by cortisol at ED $_{0(g)}$ A simple t test

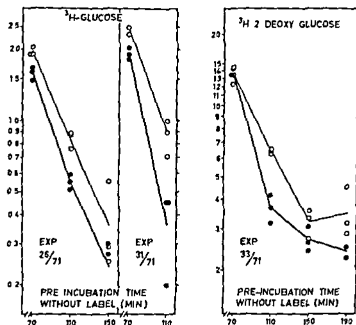


Fig 2 The effect of different pre incubation times on the 40 min uptake of ^{3}H glucose or ^{3}H-deoxy glucose in the absence (o) or prescene (•) of cortisol (1000 ng/ml) The procedure was the same as described in Methods but the cells were incubated for different times before the labelled sugar was added The curves connect the arithmetical means of each group The ordinates indicate cpm × mg^{-1}/cpm × μl^{-1}

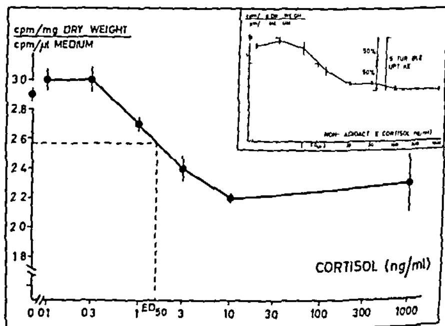


Fig 3 The dose response curve for cortisol on the inhibition of ^{3}H glucose uptake by PMN ED$_{50 (gl cose)}$ is the concentration that gives half maximal effect on the uptake The cells were incubated as described in Methods with increasing concentrations of cortisol There were 4—5 samples in each group Vertical bars indicate S E The cells were pooled from 2 rabbits A separate experiment (see *insert*) shows the dose response curve for cortisol on the inhibition of specific (saturable) ^{3}H-cortisol uptake by rabbit PMN (in the presence of 1 ng ^{3}H cortisol per ml) (for details see Simonsson 1972)

TABLE II Uptake of H glucose (cpm × mg dry weight^{-1}/cpm × µl medium^{-1}) in rabbit PMN incubated in the presence of different potent glucocorticoids The concentration of the steroids was 1000 ng/ml Figures within brackets indicate range There were 3—5 samples in each group

Experiment no Substance	14/71	15/71	48/71	49/71
Cortisol	1 7 (1 5—2 1)	2 6 (2 4—2 9)	3 1 (2 8—3 3)	3 9 (3 4—4 2)
Triamcinolone acetonide	1 3 (1 28—1 31)	2 5 (2 3—2 6)	3 2 (2 7—3 7)	
Dexamethasone			3 1 (2 7—3 3)	
9 F prednisolone			2 8 (2 5—3 2)	
Triamcinolone				3 8 (3 6—4 1)

showed that progesterone even at 300 ng/ml produced a significantly lower depression ($p < 0\,025$) than cortisol at $ED_{50(glucose)}$ Thus its relative potency is certainly less than 0 01

Fig 4 shows that tetrahydrocortisone and the cortisol isomer 11-epicortisol which both are biologically inactive as glucocorticoids have very low affinity to the glucocorticoid uptake process and also a very low effect on the ^{3}H glucose uptake It should be emphasized however that the exact figures for the relative potencies have not been determined for these steroids A high affinity to the glucocorticoid uptake process with two exceptions is accompanied by a great inhibiting potency on the ^{3}H glucose uptake

TABLE III The variations in $ED_{50(glucose)}$ for cortisol For details see text

Experiment no	Animals	$ED_{50(glucose)}$
16/71	73+79	1 6
17/71	76+80	2 9
19/71	73+79	2 6
20/71	76+80	0 64
38/71	78+87	1 1
39/71	78+87	1 8
41/71	80+88+89+91	0 3
4 71	80+88+89	0 9
43/71	87+90	1 8
44/71	80+88+91	1 4
46/71	88+89	3 7
48/71	78+80	1 4
49/71	88+89	4 5
2/72	88+93	3 0
3/72	79+81+92	2 0
	Mean ± S D	2 0 ± 1 2

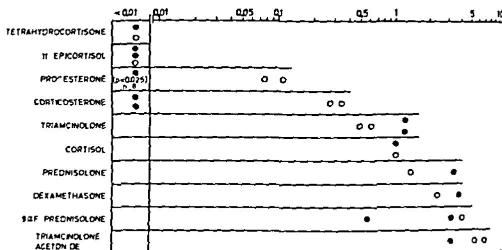


Fig 4 The relative potency (*versus* cortisol) of some steroids to depress the ^{3}H glucose uptake in rabbit PMN (●) For details see text Open symbols (○) indicate the relative affinity (*versus* cortisol) of the steroids to the specific glucocorticoid uptake process in these cells (for details see Simonsson 1972)

TABLE IV The effect on ^{3}H glucose uptake of cortisol progesterone and the combination of both. The cells were incubated and processed as described in Methods Controls were without steroid except in two cases The cells were from 6 rabbits In 4 expts all combinations of steroids were run simultaneously In 1 expt the high doses of cortisol and progesterone were not included but the rest were run simultaneously Each expt was used as a statistical unit

* = $p < 0.05$ ** = $p < 0.01$

Steroids	ng/ml	Number of expts	Uptake of ^{3}H glucose $100 \times \left(\frac{\text{Experimental} - \text{Control}}{\text{control}}\right) \pm$ S E. Cortisol added to controls	No steroid added to controls
Cortisol	3	5		-19.9 ± 5.8
Cortisol	10	4		-20.0 ± 5.2
Progesterone	300	5		-4.4 ± 5.6
Progesterone	1000	4		-4.5 ± 6.2
Cortisol + Progesterone	3 300	5	$+16.4 \pm 4.4$* (controls cortisol 3)	-7.0 ± 5.7
Cortisol + Progesterone	10 1000	4	$+18.5 \pm 3.6$ * (control cortisol 10)	-5.3 ± 4.6

Progesterone is one of the exceptions Its relatively high affinity to the glucocorticoid uptake process is in contrast to its low activity on ^{3}H glucose uptake. If the specific *glucocorticoid uptake in the PMN's corresponds* to an interaction with a specific glucocorticoid receptor acting as an intermediary in the glucocorticoid effect on the ^{3}H glucose uptake then progesterone should act as an anti glucocorticoid *i e* in high concentrations it should keep cortisol away from the uptake process

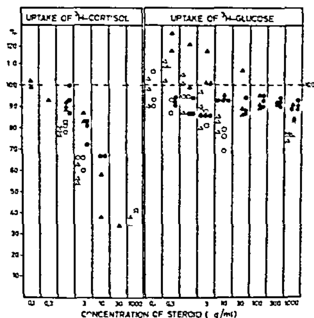


Fig 5 Effect of cortisol (open symbols) and corticosterone (closed symbols) on uptake of ^{3}H-cortisol and ^{3}H-glucose in rabbit PMN The uptake is expressed as % of the mean of the untreated controls The cells were incubated with ^{3}H glucose as described in Methods or for 25 min at 37 C with ^{3}H-cortisol (1 ng ml) All samples were then processed as described in Methods. Each sample is shown with a symbol. In the ^{3}H-cortisol expts the triangles are from 8/71 and the circles from 11/71 The triangles in the ^{3}H-glucose expts are from 2/72 and the circles from 3/72. The figure shows that corticosterone at 30 ng/ml has depressed all the specific (saturable) ^{3}H-cortisol uptake while on the ^{3}H-glucose uptake it never reaches the cortisol 1000 ng/ml level.

and thereby block its metabolic effect That this is the case is shown in Table IV where progesterone in high concentration blocks the effect of cortisol on the ^{3}H glucose uptake

The other exception (in Fig 4) is corticosterone which contrary to progesterone is a relatively potent glucocorticoid *in vivo* Its potency (*versus* cortisol) is 0.3 in standard glucocorticoid tests (for ref see Simonsson 1972) If the specific uptake of the glucocorticoids by the PMN corresponds to a receptor mediating their effect on the ^{3}H glucose uptake then corticosterone too should depress the ^{3}H glucose uptake Fig 5 shows that corticosterone in fact depresses the ^{3}H glucose uptake but only to a limited extent decidedly less than cortisol Thus corticosterone in spite of its relatively high affinity to the glucocorticoid uptake process (left part of Fig 5) seems to be only a partial agonist on the ^{3}H glucose uptake and its effect does not even reach the ED_{50} 1 ... level for cortisol. This is the reason why its relative potency calculated from the ED_{50} based on the maximum depression caused by the other glucocorticoids is less than 0 01 (Fig 4)

Table V shows the influence on the ^{3}H-glucose uptake of steroid doses that depress 50 % of the specific ^{3}H-cortisol uptake in the presence of 1 ng radioactive cortisol (concentrations taken from Simonsson 1972) Progesterone again is inactive while most conventional glucocorticoids show a depressing potency To say whether or not 11-epicortisol corticosterone and 9α F prednisolone at this dose really depress the ^{3}H-glucose uptake needs further examination. Table V also shows the variations in per cent effect on the ^{3}H glucose uptake brought about by 2 1 ng cortisol per ml medium.

TABLE V Influence on the ³H glucose uptake by the steroid concentrations that depresses 50 % of the specific (saturable) ³H cortisol uptake (in the presence of 1 ng ³H cortisol)

Substance	A ED 50 ³H cortisol uptake (ng/ml)	% inhibition of total ³H glucose uptake at concentrations shown in column A									
		14/71	16/71	18/71	20/71	43/71	46/71	48/71	49/71	7/72	3/72
Tetrahydro cortisone	> 1000								−8 (1000 ng/ml)		
11 epicortisol	1000										9
Progesterone	19										0
Corticosterone	6 0									1	10
Triamcinolone	3 7			11	29						
Cortisol	2 1	20	17		30	12	8	11		11	10
Prednisolone	1 5									17	
Dexamethasone	1 0					12	11				
9αF prednisolone	0 65						9	−8			
Triamcinolone acetonide	0 44	26	19	24	28						

Discussion

Rabbit PMN metabolize glucose (Stjernholm *et al* 1969) In the present work it was not possible to estimate the glucose consumption since the efflux of tritium from the cells was not measured The glucose is taken up by the PMN by a process which has a limited capacity the amount of tritium taken up was reduced by about 60 % by doubling the concentration of non radioactive glucose in the medium (Table I) Glucose and the non utilizable ³H deoxy glucose possibly use the same transport process into the cells since the uptake of labelled ³H deoxy glucose was similarly depressed by non radioactive glucose (Table I)

Incubation with probably physiological concentrations (Ganjam 1970) of cortisol (10^{-9}—10^{-8} M) reduced the cellular uptake of tritium from ³H glucose and ³H deoxy glucose within 2 h (Figs 1 and 2) This was expected since glucocorticoids *in vitro* inhibit uptake of glucose or labelled glucose by mouse fibroblasts (Cray 1971) mouse ear strips (Overell *et al* 1960 Matsui and Plager 1969) mouse lymphosarcoma (Rosen *et al* 1970 a and b) and rat thymus cell (Morita and Munck 1964) It has also been reported that cortisol (10^{-6}—10^{-4} M) reduces the *in vitro* glucose utilization of human polymorphonuclear leukocytes (Rauch 1961) The effect in these tissues was observed after a latency of 0 25—2 h and was in the range 12—31 % Thus the small inhibiting effect of cortisol on ³H glucose uptake in the present work is not dissimilar from its effect in other tissue It should also be noted that the cortisol concentrations used in the present work are very low and probably physiological It therefore would have been remarkable if the effect had been much greater

In the above papers there are mainly two opinions on where the glucocorticoids interfere in glucose metabolism either on the transport of glucose into the cells or on the phosphorylation to glucose 6 phosphate

According to the literature 2 deoxy glucose is not metabolized past the initial phosphorylation in all tissues where this has been studied (Kipnis and Cori 1969 Hochster and Quastel 1963 Rosen 1970 a Smith and Stultz 1971) Barban and Schultze (1966) however reported that it was metabolized by extracts from HeLa cells but only to a very limited extent Probably 2-deoxy glucose is a non utilizable hexose also for the rabbit PMN since this seems to be the case for human granulocytes (Kvarstein 1969) Figs 1 and 2 show that cortisol also depresses the uptake of ^{3}H-deoxy glucose Thus provided that 2-deoxy glucose is not metabolized past the initial phosphorylation since glucose and 2 deoxy glucose probably use the same uptake process into the cells it is not impossible that cortisol in the rabbit PMN's too interferes either with transport of glucose into the cells or with the phosphorylation to glucose-6-phosphate Whether this effect is primary or secondary to an interference with another pathway of metabolism cannot be judged from data presently available

In rat thymus cells Munck and Brinck Johnsen (1968) found a correlation between affinity to a glucocorticoid "receptor and the effect on glucose uptake of a variety of steroids The affinity constant for the cortisol binding process in the thymus was however 100 times lower than that in the PMN and this could explain the higher cortisol concentration (about 25—50 times) necessary to reach any effect on the glucose uptake in the thymus cells Among other reported tissues only the mouse ear strips (Overell *et al* 1960 Matsui and Plager 1969) seem to have a glucose metabolism which *in vitro* is about as sensitive to cortisol as that of the rabbit PMNs

Fig 4 shows that triamcinolone cortisol prednisolone dexamethasone 9αF prednisolone and triamcinolone acetonide which all have high affinity to the glucocorticoid uptake process in the PMN and *in vivo* are potent glucocorticoids (for ref see Simonsson 1972) were correspondingly potent inhibitors of ^{3}H glucose uptake *in vitro* This was not surprising since previous results (Simonsson 1972) have shown highly significant correlations between *in vivo* glucocorticoid activity and affinity to the specific glucocorticoid uptake process in the PMN This indicates that the specific glucocorticoid uptake in the PMN might [illegible] act as an intermediary in the glucocorticoid effect on the ^{3}H glucose uptake [illegible] uptake is to a receptor There are also other [illegible] such a connection.

Tetrahydrocortisone and [illegible] inactive as glucocorticoids [illegible] 1972 [illegible] affinity to the glucocorticoid uptake process [illegible] which is not a glucocorticoid [illegible] the ^{3}H-glucose up[illegible] affinity to the [illegible] process (Fig 4 [illegible])

similar effect of progesterone has also been reported on the cortisol induced rate of synthesis of the enzyme tyrosine amino transferase in hepatoma cells (Samuels and Tomkins 1970), the prednisolone induced elevation of alkaline phosphatase in HeLa cells (Melnykovych and Bishop 1969) (here corticosterone also had a similar effect) and the cortisol inhibition of ^{3}H uridine incorporation into RNA in thymocytes (White and Makman 1967) The concentrations used in the quoted experiments were about 10^{-5} M progesterone and about 10^{-6} M cortisol The concentrations used in the present work were lower, 10^{-6} M and 8×10^{-9} M respectively A relatively high affinity to the glucocorticoid receptor and a low effect on glucose uptake was found for progesterone also by Munck and Brinck Johnsen (1968) they did however not test this steroid as an anti glucocorticoid

Progesterone differs from cortisol in lacking OH groups at C 11 C 17 and C-21 The absence of the OH group at C 11 seems to be an important factor in the anti cortisol effect since similar properties have also been reported for cortexolone on cortisol inhibition of glucose uptake (Munck and Brinck Johnsen 1968) and prednisolone induced elevation of alkaline phosphatase (Melnykovych and Bishop 1969) Cortexolone differs from cortisol in lacking only the OH group at C 11 Thus by eliminating the 11 OH the steroid loses all its glucocorticoid potency but still has affinity to the uptake process The absence of the 17 OH also reduces the effect on ^{3}H glucose uptake *corticosterone (which is 17 deoxy cortisol)* seems to act as a partial agonist (Fig 5) The significance of the 21 OH group cannot be interpreted at present

Finally the striking fact that the concentration of cortisol that gives half saturation of the steroid uptake (about 1 ng/ml) is very close to the ED$_{50}$ for depression of ^{3}H glucose uptake strongly suggests that the cortisol uptake is in fact physiologically important that the binding is not to a silent site but to a physiological receptor

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Extent of Engagement of Various Cardiovascular Effectors to Alterations of Carotid Sinus Pressure

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Abstract

Kendrick E B Öberg and G Wennergren *Extent of engagement of various cardiovascular effectors to alterations of carotid sinus pressure* Acta physiol scand 1972 *86* 410—418

Reflex adjustments of blood pressure heart rate skeletal muscle and renal resistance vessels to changes in intrasinusal pressure were recorded and the respective sinus pressure—reflex response curves plotted In this way one could evaluate whether the various individual reflex arcs which together constitute the baroreceptor reflex control of the circulation display "threshold" response and maximal sensitivities respectively at identical sinus pressures —The curve describing the blood pressure muscle and renal resistance vessel responses were found to follow identical courses while the heart rate response curve was displaced to the right of the others This discrepancy is however in all probability a mere consequence of the peculiar neuroeffector characteristics in the heart The individual components of the baroreceptor reflex therefore seem to be recruited simultaneously when the sinus pressure is increased and there is thus nothing to indicate that afferents from low and high "threshold" baroreceptors respectively are preferentially distributed to different neuron pools in the bulbar cardiovascular centres —A displacement of the response curves to the right was observed when

a) non pulsating sinus pressures were used instead of pulsating

b) sinus pressures alterations were induced by lowering the pressure from higher levels instead of elevating it

c) arterial PCO_2 was increased

The reflex function of the arterial baroreceptors is usually described in terms of direction and extent of change in mean arterial blood pressure or and heart rate caused by a given change in mean pressure or pulse amplitude in isolated carotid sinus preparations (e g Koch 1931 Heymans *et al* 1931 Gero och Gerova 1967) Such studies indicate that the maximal sensitivity of the baroreceptor reflex mechanism i e the steepest part of the curve relating changes in systematic pressure to changes in intrasinusal pressure is found around 120 mm Hg while variations of sinus pressure below ca 60 mm Hg or above 210 mm Hg produce no reflex blood pressure responses Koch 1931 Heymans and Neil 1958

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The blood pressure responses to variations in baroreceptor activity are the net result of an altered activity of several different cardiovascular effectors such as the heart and the resistance and capacitance vessels of the various systemic circuits One may therefore look upon the baroreceptor reflex as consisting of several components with their respective efferent pathways in the sympathetic vasoconstrictor nerves to the different vascular bed and in the vagal and sympathetic fibres to the heart It is not known however whether these separate reflex mechanisms exhibit the same threshold intrasinusal pressure at which they become activated Nor is it known whether the slopes of the respective curves relating sinusal pressure to effector reflex response differ Discrepancies in these respects would for example be expected to occur if afferents from low and high threshold receptors respectively were to some extent differently oriented to the separate central neuron pool controlling the efferent vasomotor outflow to the various parts of the circulatory system

In case the mentioned individual reflex mechanisms constituting the baroreceptor reflex differ with regard to sensitivity the engagement of the various circulatory effectors to *e g* carotid occlusion should vary in magnitude depending on the initial level of intrasinusal pressure and the extent of reduction of this pressure resulting from the occlusion The non uniformity of the efferent vasomotor outflow to various cardiovascular target organs with this manœvre *(e g* Löfving 1961 Hadjiminas and Öberg 1968) could then be partly ascribed to a differentiation at the receptor level

The present study was undertaken to analyse this problem Reflex adjustments of blood pressure heart rate and flow resistance in skeletal muscle and kidney have been simultaneously followed when the pressure in isolated sinus regions was altered in well defined steps From the data intrasinusal pressure—reflex response curves for the mentioned variables were constructed

Methods

Experiments were performed on 25 cats anesthetized with chloralo e 30—50 mg/kg b w A tracheal cannula was inserted and the carotid sinus areas on both sides were exposed The main arterial branches from the carotid artery in the sinus region were ligated but no attempts were made to obtain a complete isolation of the sinus area The aortic nerves on both sides were identified and cut at their junction with the superior laryngeal nerves In some animals where the aortic nerves could not be identified with certainty both vagal nerves were cut in the neck

The abdomen was opened and the intestine the omentum and the spleen were removed The superior mesenteric artery was cannulated in central direction with a polyetylene tubing passing a Harvard finger pump and connected in cephalad direction to the common carotid arteries The carotid sinus areas were perfused through this exteriorized loop A Windkessel reservoir was connected to the loop via a rubber T tube so that the sinus regions could be exposed to either pulsatile or nonpulsatile pressures from the pump The external carotid arteries were cannulated in central direction and connected via a polyethylene tubing to the right external jugular vein By adjustments of a screw clamp on this tubing the outflow resistance from the sinus regions and hence the intrasinusal pressure could be varied over a wide range The intrasinusal pressure was recorded with a Statham transducer from a side branch on the outflow tubing close to the sinus region

Blood pressure was measured from a femoral or a brachial artery with a Statham transducer and recorded on a Grass Model 5 Polygraph recorder Heart rate was monitored by a tachograph which was triggered by the rapid systolic rise of the arterial blood pressure Blood

flow from the left kidney was measured by cannulating the renal vein and passing the outflow through a drop recorder operating an ordinate writer on the Polygraph The blood was returned to the animal through the superior mesenteric vein Calf muscle blood flow was similarly measured as the venous outflow from the popliteal vein The blood was returned through the opposite femoral vein The paw was excluded from circulation by mass ligatures Flow resistance in the two vascular beds was calculated from the pressure/flow relationship The arterial blood pressure could be maintained constant during the experimental procedures by connecting the arterial side of the circulation via one femoral artery to a pressure compensator

Heparin (5 mg/kg b w) was used as an anticoagulant Animals with intact vagi were usually given atropine (0 3—0 5 mg/kg b w) during the course of the experiment In some animals artificial respiration was administered and the animals curarized with Flaxedil (3—5 mg/kg b w)

Experimental procedures The speed of the perfusion pump was initially adjusted so that with complete occlusion of the outflow tubing from the sinus regions a high intrasinusal pressure (usually 200—230 mm Hg) was obtained By adjustments of a screw clamp on the outflow tubing the intrasinusal pressure could be varied from this high level down to 5—10 mm Hg By excluding or including the Windkessel reservoir in the perfusion system either pulsatile or non pulsatile intrasinusal pressures could be used. The pulse frequency was then maintained constant at a value between 150 and 200 pulses/min in each experiment The pulse amplitude varied however dependent on the extent of outflow resistance from the sinus regions from a maximum value of around 50 mm Hg when the outflow tubing was completely obstructed to a minimum value of around 20 mm Hg when the screw clamp on the outflow tubing was completely released When pulsatile pressures were used the mean pressure was estimated and used in the treatment of the data Each intrasinusal pressure level was maintained for a period of one minute during which new steady state levels of blood pressure heart rate and regional blood flows were usually established Repeated runs were done in each animal In approximately every second run the arterial blood pressure was kept constant by introducing the pressure compensator In this way pressure-dependent alterations of vessel tone was avoided However the flow resistance changes were not significantly different whether the arterial pressure was allowed to vary or not and the flow resistance data obtained from the two types of experiment have therefore been treated together The reflex inhibitory responses of the various circulatory variables were expressed as per cent of maximal responses which were obtained at sinus pressures of 200—230 mm Hg

Results

Fig 1 summarizes the results from all experiments where the intrasinusal pressure (pulsating was elevated in steps from very low to high levels The data are presented as curves relating the intrasinusal pressure to the reflex response which is expressed as per cent of maximal inhibition obtained at 200—230 mm Hg mean intrasinusal pressure The curves represent the mean values from all experiments in this series Elevations of the intrasinusal pressure produced as expected progressive reductions of blood pressure heart rate and flow resistance in the kidney and in the skeletal muscle The lowest intrasinusal pressure at which discernible reflex effects on the blood pressure renal and muscle flow resistances were observed i e the threshold value appears to be about 20 mm Hg and the same for all the mentioned variables The relative magnitude of the reflex responses to any given level of intrasinusal pressures is also identical The intrasinusal pressure reflex response curves for the mentioned variables thus have an identical course

On the other hand the threshold intrasinusal pressure at which clearcut reductions of heart rate were obtained was decidedly higher around 40 mm Hg The relative magnitude of the reflex heart responses was also significantly less than the blood

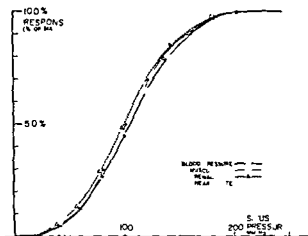


Fig 1 Carotid sinus pressure—reflex response curves for blood pressure heart rate and muscle and renal resistance vessels obtained when the sinus pressure (pulsating) was step wise increased from low to high values The data for blood pressure heart rate and muscle resistance vessels represent mean values from 20 experiments on 11 cats The renal data represent mean values from 11 of these experiments (5 cats) Vertical bars indicate 2 S E

pressure or vascular responses at any given intrasinusal pressure below approximately 200 mm Hg The intrasinusal pressure—reflex heart rate response curve is therefore displaced to the right of the other response curves The maximal sensitivity of the reflex blood pressure renal and skeletal muscle responses fell between 80—120 mm Hg while the heart rate responses showed maximal sensitivity in a somewhat higher sinusal pressure range between 100—160 mm Hg

Alterations of the sinus pressure thus produced parallel changes of renal and muscle flow resistance when expressed in per cent of the maximum decrease of resistance in the mentioned two circuits If on the other hand the reflex responses are expressed either in absolute terms or as per cent decrease of flow resistance from control they differed markedly in the 2 beds Thus a given elevation of sinus pressure regularly produced a more pronounced reflex decrease in flow resistance in the calf muscles than in the kidney In the experiments described in Fig 1 the maximum reflex decrease of flow resistance averaged 53 % in skeletal muscles but only 24 % in the kidney The maximum reflex decrease of blood pressure averaged 61 %

The maximal range of reflex reduction of heart rate when measured in the steady state situation (see below) was usually small and averaged only 11 % in the experiments shown in Fig 1 The pattern of the heart rate response differed markedly depending on whether the vagi were left intact or cut or on whether atropine was given or not With intact vagi elevation of the sinus pressure induced an initially very marked slowing of the heart The heart rate gradually recovered however so that within some 20—30 s a steady state situation with only a moderately reduced heart rate was attained When the vagi were cut or the animals atropinized the reflex slowing of the heart to a rise of intrasinusal pressure developed gradually and attained a steady state level first after about 30—40 s This steady state heart rate response which did not differ significantly whether the efferent vagal innervation

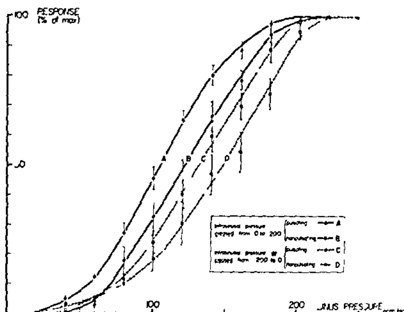


Fig 2 Comparisons of the sinus pressure — reflex blood pressure responses obtained with varying experimental procedures i e when the sinus pressure is on one hand stepwise increased and on the other stepwise decreased and when the sinus pressure is either pulsating or non pulsating Curves A and C represent mean values from 20 runs (11 cats curves B and D mean values from 18 expts (9 cats) Vertical bars represent 2 S E

was intact or not was used for the heart rate intrasinusal pressure data shown in Fig 1

The relationship between intrasinusal pressure and the relative magnitude of the reflex responses as shown in Fig 1 varied depending upon the experimental procedure Thus if the sinus regions were exposed to nonpulsatile pressures the response curves were as expected shifted to the right of those obtained with pulsating pressures This is demonstrated in Fig 2 where for the sake of clarity only the reflex blood pressure responses are shown The threshold sinus pressure at which a discernible blood pressure response could first be observed is consequently somewhat higher with non pulsating than with pulsating intrasinusal pressures Even if the maximal fall in blood pressure was essentially the same in the two situations a higher non pulsating pressure was required to produce this maximal response (compare curves A and B) Maximal sensitivity of the reflex i e the maximal steepness of the curves did not differ if pulsating or non pulsating sinus pressures were used but occurred in different intrasinusal pressure ranges namely at about 115—150 mm Hg for non pulsating and at about 80—120 mm Hg for pulsating pressures A shift from pulsating to non pulsating intrasinusal pressure will thus lead to a reflex rise of blood pressure within the whole range of intrasinusal pressures but maximal effects of depulsation seem to occur in the region of around 120 mm Hg In this sinus pressure region the baroreceptor reflex is evidently most sensitive not only to changes in mean pressure but also to alterations in pulse pressure

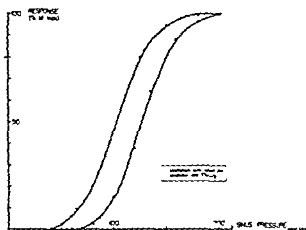


Fig 3 Comparison of the sinus pressure—reflex blood pressure response curves obtained when the animal is ventilated with air and with a gas mixture containing 7 % CO_2 in O_2 respectively The data are obtained from 1 expt.

Fig 2 also demonstrates that the magnitude of the reflex response obtained at a given intrasinusal pressure is also dependent on whether this pressure level is reached by elevating the pressure from a lower level or by lowering the pressure from a higher value (compare curves A and C) In the latter situation the reflex inhibition was less effective i e the blood pressure attained a higher value at any given pressure in the carotid sinus

Alterations of the acid base relationships in the body fluids produced by ventilating the animal with high CO_2-gas mixtures also caused a shift of the intrasinusal pressure-effector response curves to the right This is demonstrated in Fig 3 which shows the blood pressure responses to a stepwise increase of the intrasinusal pressure (pulsating) in one animal under normal conditions and when the animal inhaled a gas-mixture containing 7 % CO in O A given rise of intrasinusal pressure is seen to cause far less reduction of blood pressure when PCO was high

Even if the response curves could thus be markedly shifted with alterations in the experimental procedures, the mutual relation between the response curve for the various effectors remained the same as shown in Fig 1 Thus the curves of blood pressure muscle flow resistance and renal flow resistance responses had in general an identical course while the heart rate response curve consistantly fell to the right of the others irrespective of whether the pressure was pulsating or not and irrespective of whether the intrasinusal pressure was gradually elevated or lowered

Discussion

The present study was undertaken to compare the intrasinusal pressure—reflex response characteristics of the different individual components which together constitute the baroreceptor reflex The results indicate that the threshold reflex responses of the resistance vessels in skeletal muscle and kidney were obtained at identical intrasinusal pressures and that the relative magnitude of these responses remained essentially the same over the entire range of intrasinu

changes The sinus pressure reflex response curves for these 2 vascular beds were thus identical and they coincided with the blood pressure response curve On the other hand reflex changes in heart rate occurred at decidedly higher "threshold" intra sinusal pressure levels and the curve relating the sinus pressure to the reflex heart rate change was throughout placed to the right of the response curves for blood pressure, renal and skeletal muscle resistance vessels

It was presently assumed that the augmented sympathetic activity present at low intra sinusal pressures and revealed as high levels of blood pressure heart rate and vessel tone was solely the effect of a complete withdrawal of baroreceptor restraint on the medullar cardiovascular centres and not also due to a concomittant excitatory influence from the carotid body chemoreceptors This assumption was based on the finding that essentially no changes in blood pressure or respiration occurred when the sinus pressure, and hence the perfusion pressure for the carotid body was varied within the range between 40—10 mm Hg Only when sinus pressure was reduced to below 10 mm Hg there were in a few cases evidence for a chemoreceptor activation in terms of a sudden rise of blood pressure and an increase of ventilation. It is however possible that a slight chemoreceptor activity prevails also at somewhat higher pressures and that the slight reduction of blood pressure in response to a rise of the sinus pressure within the lower intrasinusal pressure range is due to the elimination of a certain chemoreceptor influence rather than to a stimulation of baroreceptors The threshold pressures for the baroreceptor reflex may therefore be somewhat higher than those found in the present series of experiments

The course of the curve relating effector response to the sinus pressure is determined not only by the extent of the reflexly governed activity in the efferent visceromotor fibres but also by the frequency response characteristics of the respective neuroeffectors It is known (Folkow *et al* 1956 Lindgren and Manning 1963) that very pronounced heart rate responses are obtained with very low rates of stimulation of the sympathetic accelerance fibres Thus 80 of the maximal response occurs already with stimulation frequencies around 3—4 Hz and only small changes in heart rate are obtained when the frequency is altered in the range above 4 imp s This implies that the curve relating heart rate responses to the rate of stimulation of the accelerance fibres is very steep in the low frequency range (between 0—4 imp s but almost flat in higher frequency ranges One would therefore expect that clearcut reflex changes in heart rate in response to elevations of the sinus pressure would occur first when the sinus pressure is high enough to produce a substantial reflex reduction of accelerance fibre activity In contrast the frequency response curve for the skeletal muscle resistance vessels is less steep reaching a plateau first at a stimulation rate around 10—12 Hz Mellander 1960) The configuration of the frequency response curve for renal resistance vessels resembles closely that for a muscle vascular bed Celander 1954 Therefore clearcut reflex resistance vessel responses in the two beds should occur even at a moderate reflex inhibition of vasoconstrictor fibre discharge i e already at fairly low intrasinusal pressure levels The fact that the sinus pressure reflex heart rate response curve is

displaced to the right of those for the renal and skeletal muscle resistance vessels is therefore probably a result of such differences in neuroeffector characteristics and does not necessitate e g a preferential distribution of high threshold baroreceptor afferents to the cardiac neurons in the medullar cardiovascular centres One may therefore conclude that afferents from baroreceptors with different thresholds are distributed fairly evenly to the various central vasomotor neurons and that the non uniform activation of the various sets of visceromotor fibres upon e g carotid occlusion is rather due to a differentiation within the vasomotor centre itself

This circumstance does not however contradict the recent observation that medullated and non medullated afferents in the sinus and aortic nerves seem to differ with regard to their distribution among the various neuron pools in the medullar cardiovascular centres where the non medullated fibres show a greater tendency for an orientation towards the cardiac neurons (Kendrick *et al* 1972 b)

The usually rather small reflex adjustments of the renal circulation when the baro receptors are activated has been ascribed to a particularly low inherent activity in renal vasomotor neurons in the vasomotor centre (Folkow Johansson and Lofving 1961 Kendrick Öberg and Wennergren 1972 a) One may therefore *a priori* have expected to find a maximal inhibitory response in this vascular bed already at a fairly moderate increase of baroreceptor inhibitory influence e g at rather low intrasinusal pressures and that therefore the renal response curve should be dis placed to the left of e g the response curve for muscle resistance vessels This was obviously not the case however, as the two curves showed an almost identical course The implication of this finding is at present difficult to comprehend but it may suggest that a smaller number of baroreceptor afferents are distributed to the central vasomotor neurons controlling the renal vessels than to those controlling e g the skeletal muscle vascular bed

A displacement of the heart rate response curve to the right of e g the blood pressure response curve as illustrated in Fig 1 is also apparent from the data presented by Lindgren and Manning 1965 Similarly the curve describing alterations in cardiac output to changes in intrasinusal pressure also seems to be displaced to the right of the blood pressure response curve when judged from the results obtained by Kumada and Iriuchijima (1965)

Brender and Webb Peploe (1969) recently studied reflex adjustments of aortic pressure iliac artery perfusion pressure and venous pressure in the isovolumetric spleen when the pressure in isolated sinus preparations was altered All three vari ables showed the most marked responses to changes in sinus pressure in the same sinus pressure range (110—160 mm Hg) but whether they differed with respect to threshold intrasinusal pressure is however not evident from this study

The differences in the course of the curves relating intrasinusal pressure to reflex response depending upon whether the intrasinusal pressure was gradually lowered or raised and depending on whether pulsating or non pulsating intrasinusal pressure were used is most reasonably explained by an adaptation of the reflex mechanism This adaptation takes place partly in the receptors themselves a phe

known from electrophysiological studies of impulse activity in baroreceptor afferents (*e g* Landgren 1952) However a certain adaptation evidently occurs in the central synapses of the baroreceptor reflex pathway (Richter Keck and Seller 1970) In addition during a period with very strong baroreceptor stimulation causing an extensive reflex blood pressure fall the possibility exists that the cerebral blood flow becomes so reduced that the consequent change in the chemical environment in the vasomotor centre enhances its spontaneous activity markedly and thus makes inhibitory influences less effective The shift of the response curve to the right after inhalation of high CO mixtures implying a smaller inhibitory response to a given intra sinusal pressure level indicates that a disturbance of the chemical composition of the blood can indeed alter the responsiveness of the vasomotor centre

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Plasma Kinins and Adrenergic Vasodilatation in the Submandibular Salivary Gland of the Cat

By
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Abstract

GAUTVIK K M M KRIZ and K LUND LARSEN *Plasma kinins and adrenergic vasodilatation in the submandibular salivary gland of the cat* Acta physiol scand 1972 86 419—426

The submandibular salivary gland of the cat has been perfused *in situ* at constant volume inflow and in such a way that perfusion periods with kininogen-containing and with kininogen free perfusates alternated The gland was stimulated with close arterial injections of noradrenaline and the secretory and vascular responses recorded Doses of noradrenaline which caused vasodilatation or no vascular effects when cat kininogen was present in the perfusate induced brisk vasoconstrictor responses in the same gland when a kininogen free perfusate was used The vasoconstrictor responses could be gradually reduced and finally abolished as the gland was reloaded with cat kininogen Glands which had undergone repeated stimulations with noradrenaline had a markedly lower content of kallikrein than had the contralateral unstimulated organ It is concluded that during adrenergic stimulation of the submandibular salivary gland kallikrein is released into the interstitial space where it induces formation of plasma kinins The plasma kinins formed cause vasodilatation and they probably play an important role in the vasodilator response observed after a period with activation through the sympathetic nerves

Stimulation of the sympathetic nerves to the submandibular salivary gland of the cat causes secretion of saliva contraction of myoepithelial cells and vasoconstriction (Emmelin 1955 Burgen and Emmelin 1961 Emmelin and Engstrom 1960 Emmelin Garrett and Ohlin 1968 1969) When nerve stimulation has been maintained for some time or when it is ended the vasoconstriction is followed by a vasodilatation This socalled sympathetic after dilatation cannot be explained as a simple reactive hyperaemia since the vasodilator effect of a comparable period of circulatory arrest is much smaller (Hilton and Lewis 1955 a) During the years several hypotheses have been proposed in order to explain this phenomenon Carlson (1907) suggested that specific dilator fibres were responsible for the vasodilatation He was later opposed by Barcroft and Piper (1912) who presented evidence that the vasodilatation associated with secretory activity induced by sympathetic was due to release of metabolites from gland cells More recently Barc

has been extended by Hilton and Lewis (1956) who suggested that the chemical mediator was a plasma kinin forming enzyme kallikrein. This enzyme could be recovered in glandular perfusate during activation of the organ by sympathetic amines. Bhoola *et al.* (1965) however, claimed that the vasodilatation was mediated through β adrenergic receptors in the glandular vasculature. They were able to show that β adrenergic blockade reduced or abolished the sympathetic after-dilatation whereas it left the initial vasoconstriction unaffected.

Adrenergic activation of the gland causes secretion of saliva which has a high concentration of kallikrein and there is a close correlation between the degree of secretion and the degree of sympathetic after vasodilatation (Beilenson, Schachter and Smaje 1968, Emmelin and Engstrom 1960). From earlier work it is known that the vasodilatation which is induced in the cat submandibular gland by stimulation of the chorda tympani is mediated partly through vasodilator nerve fibres and partly through plasma kinins formed in the active organ (Hilton and Lewis 1955 a, b, Gautvik 1970 a, b, Gautvik, Hilton and Torres 1970, Karpinski, Barton and Schachter 1971). On the basis of these reports we decided to examine the relationship between the plasma kinin formation and the vascular responses in glands activated by noradrenaline.*

Materials and Methods

The experiments were carried out on the *in vivo* perfused submandibular salivary gland. Cats (2.5—5.0 kg) of both sexes were anesthetized with i.p. injections of pentobarbitone (30—40 mg/kg). The arterial and venous blood supplies to the gland were completely isolated. The gland was perfused through the cannulated lingual artery at a constant volume inflow. A peristaltic pump (Model 500—1200 M, Harvard Apparatus, Dover, Mass.) was used and the flow rate adjusted to provide a venous outflow equal to that from the resting gland. The perfusion pressure was recorded with a Statham pressure transducer (P 23 De) which was connected via a 259 DC carrier preamplifier to a multichannel recorder. As the flow rate was kept constant changes in the resistance of the intraglandular vessels could be observed through changes in the perfusion pressure. The perfusion arrangement has been described in detail earlier (Gautvik 1970 a). During each perfusion period the common carotid artery was temporarily clamped.

Venous outflow was recorded by a photo-electric dropcounter as described by Gautvik (1970 a).

The perfusates used consisted of

a) washed homologous cat red blood cells resuspended in normal cat plasma (normal perfusate)

b) washed homologous cat red blood cells resuspended in horse plasma which had been adsorbed with cat red blood cells (kininogen free perfusate). The preparation of the perfusates has been described earlier (Gautvik 1970 b).

Adrenergic stimulation of the gland. During isolation of the arterial supply to the gland the sympathetic nerves running along the arteries suffered damage. Adrenergic stimulation was therefore carried out by injections of noradrenaline (Norsk Astra A/S, Oslo) close to the place where the perfusion tubing entered the lingual artery.

Saliva secretion. The submandibular ducts were exposed and cannulated. Saliva secretion was recorded by a photoelectric drop-counter and also by weighing the amount of fluid secreted.

Nerve stimulation. The chordo-lingual nerve and the sympathetic nerves in the neck were dissected free, cut and the distal ends connected to a platinum fluid electrode. The indifferent electrode was placed on the leg. The nerves were stimulated supramaximally (9—14 V) with square wave pulses of 1 ms duration and at frequencies of 20/s.

* A preliminary report of this work has been given at the Symposium in Oral Physiology, Stockholm, Sweden, August 1971.

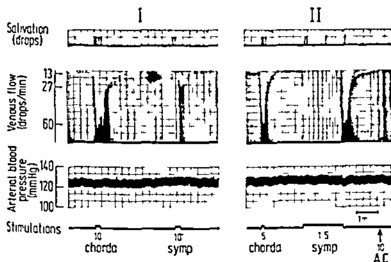


Fig 1 Typical secretory and vascular responses to stimulation of the parasympathetic and sympathetic nerves to the cat submandibular salivary gland The gland had its normal circulation and the blood flow was measured by recording the venous outflow by means of a photo-electric drop counter (se Methods) The fall of one drop of saliva or of blood is indicated by a vertical line The nerves were stimulated supramaximally at signals The circulation to the gland was arrested for 10 s by clamping the common carotid artery at (A C)

Measurements of glandular kallikrein The total content of plasma kinin forming enzyme in glandular homogenate (kallikrein activity) was measured on the isolated rat uterus preparation as described by Gautvik (1969) A partly purified preparation of cat kininogen was used as substrate (Jacobsen 1966) and the kinin activity was expressed in terms of bradykinin equivalents Synthetic bradykinin (BRS 640 Sandoz Basel Switzerland) was used as a standard

BAEE esterase activity It had earlier been shown that the major part of the BAEE splitting activity in this gland was due to kallikrein activity (Gautvik Nustad and Vystyd 1969) The hydrolysis of α N Benzoyl L-Arginine Ethyl Ester (BAEE) was used as an additional quantitative measure for the kallikrein activity in glandular homogenate as described by Gautvik Kriz and Lund Larsen (1972)

Results

Parasympathetic and sympathetic nerves stimulation in the submandibular salivary gland The normal vascular and secretory reactions of a gland to stimulation of the chordo-lingual nerve and of the sympathetic chain are illustrated in Fig 1 The gland is perfused through its normal circulation and secretion of saliva and venous outflow are recorded as described in Methods In part I of the illustration are compared the secretory and vascular responses to 10 s of parasympathetic and sympathetic nerve stimulation respectively Stimulation of the chordo lingual nerve produces a conspicuous increase in venous outflow accompanied by a lively salivation Stimulation of the sympathetic nerve fibres however causes a vasoconstriction which is followed by a marked increase in blood flow when the stimulation The production of saliva on sympathetic stimulation was small compared t

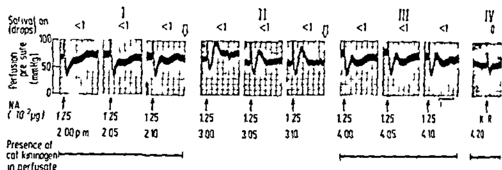


Fig. 2. Secretory and vascular responses caused by repeated injections of noradrenaline (NA) in the cat submandibular salivary gland. The gland was perfused at a constant volume inflow, and the perfusate altered with respect to the presence of substrate for cat glandular kallikrein (cat kininogen) in the four perfusion periods shown. A change in the perfusion pressure below or above the mean pressure indicates a vasodilatation or a vasoconstriction respectively. The venous outflow was in this experiment 0.25 ml/min. The presence of cat kininogen in perfusate is indicated as is the points of NA injection. In perfusion period IV is demonstrated the control vascular response to injection of an equal volume of Krebs Ringer solution. Salivation is recorded as in Fig. 1. Between each perfusion period the normal circulation through the gland was re-established (⇕).

seen on stimulation of the chordo-lingual nerve. Part II of the illustration shows the effect of markedly prolonging the period during which sympathetic nerves are stimulated. The so-called "after-vasodilatation" then reached the same level as the vasodilatation caused by a 5 s stimulation of the chordo-lingual nerve. Towards the end of the sympathetic nerve stimulation an increase in blood flow can be observed indicating a reduced vasoconstriction. At the far right end of the illustration is demonstrated the reactive hyperaemia caused by a 10 s occlusion of the carotid artery (A.C.). The increase in venous outflow after this occlusion is clearly much smaller than the one seen after a similar period of sympathetic nerve stimulation.

Secretory and vascular responses to close arterial injections of noradrenaline. Administration of noradrenaline to the submandibular salivary gland causes secretory and vascular effects identical to those of sympathetic nerve stimulation. If plasma kinins are the mediators of the sympathetic after-vasodilatation, then the vascular responses to noradrenaline would be expected to change in glands where the possibility for formation of these polypeptides has been reduced. Plasma kinin formation can be greatly impaired if the submandibular salivary gland is perfused with solutions which do not contain substrate for cat submandibular kallikrein (Gautvik 1970 a). In the following experiments adsorbed horse plasma (see Methods) was used as a "kininogen-free" perfusate since cat submandibular kallikrein does not cause kinin formation when incubated with horse plasma.

The experiments were carried out in 5 cats and more than one series of perfusions were usually performed on the same organ. In each such gland perfusion period with normal and kininogen-free perfusates alternated, and the secretory and vascular responses to noradrenaline injections were recorded as described in Methods. In this way the gland served as its own control. In Fig. 2 is shown an experiment

Fig 3 Secretory and vascular responses to injections of noradrenaline in the submandibular salivary gland Gland perfused under conditions of constant volume inflow A vascular dose-response relationship to noradrenaline is shown and also the effect of a gradual reloading of the gland with cat kininogen The experiment is otherwise of the same type as the one described in Fig 2 The venous outflow in this experiment was 0.30 ml/min

in which the gland is perfused with normal perfusate during period I III and IV and with kininogen free perfusate during period II The dose of noradrenaline (NA) (1.25 × 10 μg) injected was the same throughout the experiment and it was sufficient to cause a small secretion of saliva When the injection artefact had subsided (shown in period IV) this dose of noradrenaline elicited small and equal *vasodilator responses* during perfusion period I When during perfusion period II the kininogen free perfusate was used the same dose of noradrenaline did now cause a *vasoconstrictor response* In the subsequent perfusion period III a change to normal perfusate caused the *vasodilator responses* to noradrenaline injections to reappear Between each perfusion period the animal's own circulation through the gland was restored for 15 min The dose of noradrenaline used in the present tests caused secretion of only minute volumes of saliva

A vascular dose response relationship for doses of noradrenaline of 0.675 × 10 μg and 1.25 × 10 μg is shown in Fig 3 During perfusion with normal perfusate (period I) a very small increase in perfusion pressure could be observed in response to the highest dose of noradrenaline Perfusion with a medium containing no cat kininogen was then carried out in the first part of period II The smallest dose of noradrenaline which in the previous perfusion period had no demonstrable vascular effect did now cause a brisk vasoconstriction When the dose of noradrenaline was doubled the vasoconstrictor response was increased more than 2 fold In order to see the effect of a gradual reloading of the gland with cat kininogen the kininogen free perfusate was changed back to the normal perfusate type during the same continuous perfusion This resulted in a gradual decrease in the vasoconstrictor responses to the largest dose of noradrenaline (1.25 × 10 μg)

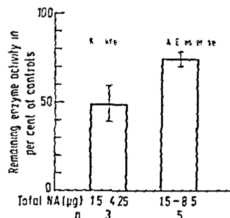


Fig. 4. Remaining kallikrein and BAEE-esterase activity in noradrenaline stimulated submandibular salivary glands. Remaining activity given in per cent of the enzyme content in the contralateral, non-stimulated control glands. The kallikrein activity was measured in three experiments using the isolated rat uterus bioassay for bradykinin. The enzyme activity was expressed in bradykinin equivalents. In addition the hydrolysis of BAEE was used as a measure for kinin forming activity. The BAEE esterase activity was expressed as µmol BAEE hydrolyzed/min g.

The changes with time of the vascular response to repeated noradrenaline injections were evaluated in control experiments where normal perfusate was used. Relatively high doses of noradrenaline (0.1 µg or more) which elicited brisk vasoconstrictor responses were chosen. During 2 hrs of perfusion a small decrease was observed in the initial response to repeated injections of such a dose. The basic features of the vascular effect were, however, never changed.

Effects of noradrenaline injections on the content of kallikrein in the submandibular salivary gland. In all experiments we used doses of noradrenaline which caused secretion of saliva. The amount of saliva secreted during one experiment was, however, usually too small for measurements of the amount of released kallikrein to be carried out. The extent of gland cell activation upon a series of noradrenaline injections was therefore evaluated by measurements of the total content of kinin forming activity in gland homogenates. The contralateral, non-stimulated gland was always used as control. The enzyme activities in the two glands from the same animal have been shown to be very similar, although considerable differences was found from the glands of one cat to those of another (Gautvik *et al.* 1972). As illustrated in Fig. 4 the noradrenaline-stimulated glands showed a mean reduction of 50 per cent (range 30—60 per cent) in their content of kallikrein. The BAEE esterase activity was also reduced in the noradrenaline stimulated glands. The mean reduction was 22 per cent (range 16—38 per cent). The total amount of noradrenaline injected during one experiment varied from 1.5 to 8.5 µg.

Discussion

Stimulation of the sympathetic nerves or administration of noradrenaline is a powerful stimulus for secretion of kallikrein. Thus glands stimulated through the sympathetic nerves show a marked reduction in their content of kinin forming enzyme (Beilenson *et al.* 1968), as did the glands stimulated with repeated injections of noradrenaline in the present study. It has earlier been shown by Hilton and Lewis

(1966) and later confirmed by Gautvik *et al* (1972) that kinin forming activity can be recovered from effluent perfusate when the gland is activated by administration of cathecholamines During the present experiments the external secretion was negligible and loss of kallikrein into saliva cannot account for the marked reduction in the glandular content of kallikrein It appears therefore that the low kallikrein level in stimulated glands is at least partly due to the release of enzyme into the interstitial space

The vascular responses to administration of noradrenaline were shown to be crucially dependent on the presence of cat kininogen in the perfusate Doses of noradrenaline which evoked vasodilatation or had no vascular effect when cat kininogen was present in perfusate did cause brisk vasoconstrictor responses when the cat kininogen had been removed The vasoconstrictor responses to noradrenaline were shown to be dose-dependent In addition the vasoconstriction was gradually diminished when the gland was reloaded with kininogen As each gland served as its own control and as this type of vascular response to noradrenaline injections did not occur during control experiments these observations cannot be explained as being caused by the perfusion arrangement It has earlier been shown in experiments performed on the lateral head of gastrocnemius that the kininogen free perfusate does not introduce any unspecific vascular responses (Gautvik 1970 b)

The following hypothesis is offered to explain the vascular events which occur in a gland stimulated by sympathetic nerve activation or by noradrenaline administration The initial vasoconstriction is caused by the action of transmittor (noradrenaline) on α adrenergic vessel receptors During gland cell stimulation kallikrein is released into the intercellular space where it splits off kinins from the kininogen present The plasma kinins formed oppose the vasoconstriction and play an important part in the sympathetic after vasodilatation The contribution to this vasodilatation by β adrenergic receptors in the glandular vessels has not yet been fully elucidated and may be difficult to evaluate since β adrenergic blockade may also inhibit release of kallikrein from the gland cells The present results strongly suggest that kallikrein is released from the gland cells and that the subsequent plasma kinin formation plays a role in the vascular responses elicited by adrenergic stimulation

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A Sulphomucopolysaccharide-Protein Complex in the Adrenergic Vesicle (Granule) Fraction from Nerves and Tissue

By

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Recent reports from our laboratory indicated the presence of sulphomucopolysaccharide (SMPS) protein complexes capable of binding biogenic amines as well as inorganic cations in granule fractions from mast cells (Uvnäs Åborg and Bergendorff 1970) thrombocytes (Åborg and Uvnas 1971) and adrenal medullary cells (Fillion Nosal and Uvnas 1971)

In this communication observations will be presented which indicate that SMPS protein complexes might also be present in adrenergic vesicle fractions from sympathetically innervated tissues peripheral sympathetic nerves and brain

Methods

In order to label any SMPS that might be present cats and dogs (of both sexes) were given $Na_2{}^{35}SO_4$ i p (8 and 20—25 mCi respectively) 24—48 h before sacrifice To obtain labelled NA in the adrenergic vesicles 200 μCi of ^{3}H DL-NA was injected i v 1 h before sacrifice In some experiments 200 μCi of ^{3}H 5 HT was given

The organ nerve or brain tissue selected was excised rinsed in ice-cold saline homogenized in isotonic sucrose and centrifuged through a continuous sucrose density gradient (Potter and Axelrod 1963) The centrifuge tubes were punctured from below and fractions of 3—5 drops were collected for determination of ^{3}H and ^{35}S activities (liquid scintillation counting) In some experiments the homogenates were pressed through Sartorius membrane filters essentially as described by Oka *et al* (1966) with a smallest pore size of 0 2 μm The precipitate after centrifugation at 125 000 × g for 30 min was used for studies on the uptake of amines or identification of SMPS

For SMPS identification the precipitate was suspended in water The insoluble residue after osmotic lysis was spun down and the SMPS isolated by hydrolysis in 0 1 M NaOH or digestion by protease and subsequent dialysis The SMPS was then identified by paper chromatography or electrophoresis or both

The storage capacity of the insoluble residue of the osmotically shocked vesicle containing fraction was determined by suspending the material in amine or cation containing solutions (Uvnas Åborg and Bergendorff 1970)

NA was determined according to Anton and Sayre (1962)

Results

Density gradient centrifugation of homogenates from the tissue studied (heart spleen abdominal sympathetic nerves (ganglia excluded) hypothalamus hypop

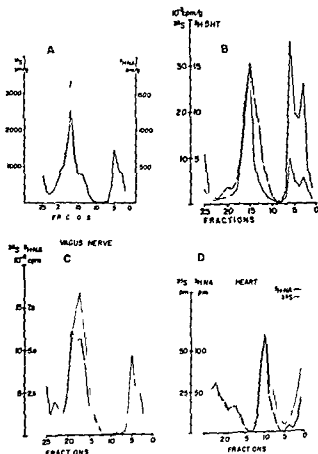


Fig 1 Distribution of ^{35}S ^{3}H Noradrenaline and ^{3}H 5 HT after centrifugation (120 000 × g 30 min 2 C) of homogenates from a) dog abdominal sympathetic chain (^{3}H NA b) dog abdominal sympathetic chain (^{3}H 5 HT) c) dog vago-sympathetic nerve (^{3}H NA) d) dog heart—right atrium (^{3}H NA) through a continuous sucrose density gradient (0 25—1 8 M) Distribution of ^{3}H NA and ^{3}H 5 HT ——— Distribution of ^{35}S

etc) resulted in similar distribution patterns for ^{35}S and ^{3}H NA activities with etc also showed similar distribution patterns for ^{35}S and ^{3}H NA with one or some experiments brain homogenates yielded several incompletely resolved activity peaks but still the distribution of the ^{35}S and ^{3}H activities coincided Identical results were obtained from cat and dog tissues

Mixed nerves such as the vago sympathetic nerve of the dog the femoral nerve etc also showed similar distribution patterns for ^{35}S and ^{3}H NA with one or some times two congruent ^{35}S and ^{3}H activity peaks (Fig 1 c) Labelling with ^{3}H 5 HT resulted in a labelling of the nerve vesicle fractions undistinguishable from the results with ^{3}H NA (Fig 1 b)

The precipitated catecholamine-containing vesicle fraction lost its catecholamine content when suspended in water The SMPS in the insoluble residue was identified as chondroitin sulphate A or a closely related substance The protein part of the SMPS protein complex has not yet been characterized

When suspended in Na or NA containing media at pH 7 the insoluble residue—containing the SMPS protein complex—took up Na and NA respectively the two uptake curves showing similar courses

Discussion

The present experiments indicate the existence of an SMPS protein complex in homogenate fractions containing adrenergic nerve vesicles (granules) Only the uptake of Na and NA in such a complex from spleen homogenate has been studied However taking into consideration previous observations on the binding properties of SMPS protein complexes from other storage granules (Uvnäs Åborg and Bergendorff 1970 Åborg and Uvnäs 1971) it seems reasonable to suppose that also the present SMPS protein complex (in adrenergic vesicles (granules)?) might have the unspecific binding properties of a cation exchange material Accordingly ^{3}H 5 HT showed a similar distribution pattern to that of H NA The observed *in vivo* exchange of NA and 5 HT in the nervous transmitter stores of the dog heart (Fillion Lluch and Uvnäs 1971) also supports the concept of an unspecific binding of amines in the adrenergic vesicles

The content of endogenous NA in the spleen vesicle fraction amounted to 3 n eq/mg while the uptake capacity was 120 n eq/mg dried material at 10 meq NA/L Even if only part of the amine binding material was identical with an SMPS protein complex in the adrenergic vesicles the uptake capacity of this complex containing material evidently seems to suffice for the storage of the neurotransmitter pool—at least the functional easily releasable pool The possible implications of these findings for the storage and release of the adrenergic neurotransmitter will be discussed elsewhere

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Analysis of Prostaglandin $F_{2\alpha}$ and Metabolites in Blood during Constant Intravenous Infusion of Prostaglandin $F_{2\alpha}$ in the Human Female

By

F Beguin M Bygdeman K Green, B Samuelsson M Toppozada and N Wiqvist

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Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and PGE_2 have been used successfully for induction of abortion either by systemic or intrauterine administration (Bygdeman and Wiqvist 1971 Karim 1971 Embrey 1971 Wiqvist Bygdeman and Toppozada 1971 Toppozada Bygdeman and Wiqvist 1971, Karim and Sharma 1971)

Gas chromatographic mass spectrometric methods for quantitative analysis of PGF_2 9α 11α dihydroxy 15 keto-prost 5 enoic acid (15 keto dihydro $PGF_{2\alpha}$) and 9α 11α 15 trihydroxy prost 5 enoic acid (dihydro $PGF_{2\alpha}$) in body fluids have recently been developed (Axen *et al* 1971 Green *et al* to be publ) This technique is based on addition of synthetic deuterated carriers to the biological sample and after extraction purification and derivatization the ratio between the protium and deuterium forms is measured in a mass spectrometer equipped with an accelerating voltage alternator

Kinetic studies after i v injection of tritium labelled PGF_2 have indicated that $PGF_{2\alpha}$ is rapidly converted into 15 keto dihydro $PGF_{2\alpha}$ and dihydro-$PGF_{2\alpha}$ The levels of these 3 compounds were determined in blood from a patient receiving i v $PGF_{2\alpha}$ at a constant rate of 75 μg min for 5 5 h Peripheral venous blood was drawn 15 min before 15 min 1 and 5 h after the start and 1 h after interruption of the administration The concentrations of PGF_2 in the collected samples were 0 1 0 7 1 3 0 9 and 0 1 ng ml blood respectively The levels of dihydro-$PGF_{2\alpha}$ were 0 1 0 9 1 0, 1 3 and 0 1 ng ml and for 15 keto-dihydro $PGF_{2\alpha}$ the concentrations were 0 2 28 32 33 and 3 6 ng ml This preliminary pharmacokinetic study demonstrated that the blood levels of these compounds were essentially constant during the infusion period and also called for further investigations to explore the degree of individual variation in the metabolism of $PGF_{2\alpha}$ as compared to the induced biological effects

TABLE I

Case no	Serum levels before and during infusion of $PGF_{2\alpha}$ (ng/ml)						Mean uterine activity Monte video Units	Episodes of side effects (diarrhea and vomiting)
	$PGF_{2\alpha}$		dihydro-$PGF_{2\alpha}$		15 keto-dihydro-$PGF_{2\alpha}$			
	before	during	before	during	before	during		
1	0 4	3 6	0 1	0 9	0	55	140	0
2	0 2	10 4	0	3 2	0	101	648	12
3	0	5 5	0 4	1 8	0 2	103	358	4
4	0 1	1 7	0 6	2 9	0	125	154	0
5	0	3 6	0	3 6	0	143	417	7
6	0 1	3 8	0 1	4 5	0	139	662	3
7	0	3 1	0	1 2	0	88	314	5
8	0	2 5	0 5	4 1	0 2	128	267	5
9	0	2 8	0 3	1 8	0	68	374	4
10	0	7 5	0	2 3	0 2	102	358	13

The study was therefore extended to a group of 10 midpregnant patients admitted to the hospital for induction of abortion Each patient received a constant infusion of $PGF_{2\alpha}$ at a rate of 75 μg/min for 10 h Peripheral venous blood (20 ml) was drawn before and 3 h after the start of the infusion and left to clot over night at 4 C The serum was isolated and 5–10 μg of each of D_4 $PGF_{2\alpha}$ D_4 15 keto-dihydro $PGF_{2\alpha}$ and D_4-dihydro $PGF_{2\alpha}$ were added (each carrier also contained tritium labelled tracer) The samples were worked up and the levels of the 3 compounds were measured in the mass spectrometer (*cf* Green *et al* to be publ) During the infusion period the incidence of side effects in terms of vomiting and diarrhea were observed and the uterine contractility was continuously recorded using a small balloon placed in the extra amniotic space and connected to a Statham pressure transducer and a Grass polygraph The results are summarized in the table

At serum levels around 2 5 ng/ml the standard deviation of the methods used is about ± 10 % while at concentrations below 0 4 ng/ml the accuracy is less satisfactory (*Green et al to be publ) Accordingly the methods used are not sensitive* enough to determine the exact preinfusion levels of these compounds

The concentration of PGF_2 ranged from 1 7 to 10 4 ng ml serum and the levels of dihydro-$PGF_{2\alpha}$ and 15 keto-dihydro-$PGF_{2\alpha}$ ranged from 0 9 to 4 5 ng/ml and from 55 to 143 ng/ml respectively The individual data demonstrated that there was a considerable variation in the rate of the initial metabolic degradation and accordingly in the inactivation of exogenous $PGF_{2\alpha}$ The data also demonstrated that a high level of PGF_2 was usually associated with a high incidence of side effects ($r = 0\ 81$ $p < 0\ 01$) whereas the correlation to the mean uterine activity was less evident ($r = 0\ 58$ $p < 0\ 05$)

The ratio between dihydro $PGF_{2\alpha}$ and $PGF_{2\alpha}$ and that between 15 keto-dihydro-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ in the different patients ranged from 0 25 to 1 7 and from 9 7 to 73 5 respectively Thus the level of the latter metabolite was approximately 10 to 75 times higher than that of $PGF_{2\alpha}$ This means that quantitation of 15 keto-dihydro-$PGF_{2\alpha}$ in blood will probably be advantageous as an indicator for the release of endogenous $PGF_{2\alpha}$ into the circulation

The levels of $PGF_{2\alpha}$ and 15 keto dihydro $PGF_{2\alpha}$ demonstrate the rapid transformation of $PGF_{2\alpha}$ into this metabolite 15 keto dihydro $PGF_{2\alpha}$ which has a considerably longer half life than $PGF_{2\alpha}$ can be further metabolized to dihydro $PGF_{2\alpha}$ (Hamberg and Samuelsson 1971 1972) The oxidation to a keto group at C-15 and saturation of the $\triangle^{13}$ double bond of a primary prostaglandin leads to compounds with considerably less biological activity on animal tissues *in vitro* However, subsequent reduction of the ketone with formation of a 15 S hydroxyl group (as in dihydro $PGF_{2\alpha}$) increases their smooth muscle stimulating properties (*cf* Samuelsson *et al* 1971 Änggard 1971) Although nothing seems to be known about the biological effects of these metabolites in humans they might contribute to the increased uterine activity and side effects observed during administration of $PGF_{2\alpha}$

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Incorporation of Reserpine into Isolated Rat Peritoneal Mast Cells and Mast Cell Granules In vitro

By

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Abstract

Gripenberg J S E Jansson and T Partanen *Incorporation of reserpine into isolated rat peritoneal mast cells and mast cell granules in vitro* Acta physiol scand 1972 86 433—443

The incorporation of reserpine into mast cells isolated from the peritoneal fluid of the rat by density gradient centrifugation was studied in vitro Reserpine was assayed fluorometrically according to Jakovlevic *et al* (1962) The results obtained suggest a passive incorporation of reserpine The incorporation took place against a concentration gradient and the drug became effectively bound to the cell Tetrabenazine prenylamine imipramine 5-hydroxytryptamine and various metabolic inhibitors did not have significant effect on the incorporation which was also independent of temperature The subcellular distribution of incorporated reserpine showed a close correlation to the distribution of 5 hydroxytryptamine suggesting that reserpine becomes almost exclusively bound to the intracellular amine storing organelles It was concluded that in such in vitro experiments with mast cells one molecule of reserpine is able to inhibit the uptake of 6 molecules of 5 hydroxytryptamine

Reserpine exerts its effect on neuronal monoamines by interfering with the ATP Mg^{++} dependent uptake mechanism of the intracellular amine storing organelles (Carlsson Hillarp and Waldeck 1963 Kirshner 1962 a b Euler and Lishajko 1963) Reserpine does also affect the 5 hydroxytryptamine (5 HT) storage and transport in rat peritoneal mast cells (Jansson 1970 a) The mechanism of action of reserpine has however, remained uncertain because reserpine does not significantly alter the normal distribution of 5 HT in mast cells (Jansson 1970 b) Reserpine is known to release 5 HT from platelets in vitro by a mechanism different from simple mole per mole displacement (Carlsson Shore and Brodie 1957) Furthermore the uptake of 5 HT into isolated membrane free mast cell granules is a passive process (Jansson 1971 a) but this uptake is effectively depressed by reserpine In a preliminary report (Jansson Gripenberg and Partanen 1971) it was reported that reserpine is incorporated into isolated mast cells against a concentration gradient and in another paper (Gripenberg and Jansson 1971) it was reported that substantial amounts of reserpine are incorporated into isolated mast cell granules Further studies on incorporation of reserpine into mast cells are reported in this paper

Material and Methods

Adult albino rats of both sexes of the Sprague Dawley strain were used. The animals were lightly anesthesized with ether and decapitated. The mixed peritoneal and pleural cells were washed out with 10 ml respectively of a modified ice-chilled Krebs-Ringer solution supplemented with calcium chloride and glucose (Eränkö and Räisänen 1966) and with 0.1% bovine serum albumin (AB Kabi) (Uvnäs and Thon 1961). After centrifugation at about 350×*g* for 5 min the cells were resuspended in fresh Krebs Ringer solution.

Isolation of mast cells. The isolation of mast cells was performed with minor modifications as described by Uvnäs and Thon (1961). 20 ml of the resuspended cells corresponding to the yield of cells from 2—4 rats was layered on the top of one 10 ml layer of 30% Ficoll (AB Pharmacia) in saline supplemented with 0.1% glucose and 0.1% bovine serum albumin. The separation of the cells was accomplished by centrifugation at room temperature at 350×*g* for 15 min. A practically pure mast cell fraction could be collected from the Ficoll layer. The cells were washed free from Ficoll with 2 changes of fresh Krebs Ringer solution and thereafter used in the experiments.

Isolation of mast cell granules. Isolation of mast cell granules was performed as described earlier (Lagunoff *et al.* 1964, Thon and Uvnäs 1966). The mast cells were washed once with 0.30 M sucrose brought to pH 6.9 by the addition of minor amounts of NaOH (about 0.02 mM). After resuspension in fresh sucrose degranulation of the cells was accomplished by freezing and thawing 3 times. A heavy sediment consisting of partially broken mast cells, debris and substantial amounts of extracellular mast cell granules was collected by centrifugation at about 350×*g* for 5 min. A practically pure mast cell granule fraction was collected from the supernatant by centrifugation at 2700×*g* for 30 min. The morphology of the sediments was checked by electron microscopy after fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 and postfixation in 1% osmium tetroxide in phosphate buffer pH 7.2. Sections were obtained on the LKB Ultrotome and stained with uranyl citrate and/or lead citrate. Philips EM 300 or AEI microscope were used.

Incubation technique. Incubations were performed in 12 ml pyrex tubes in a water bath under agitation. The incubation volume was 1.0 ml with a concentration of mast cells ranging from 0.3 to 0.8 million mast cells per ml. Reserpine was dissolved in about 10 μl of glacial acetic acid and brought to volume with absolute ethanol to give a concentration of 5×10^{-4} M. When the effect of inhibitors was studied the inhibitors were added 30 min before the addition of reserpine. The metabolic inhibitors used were dissolved in absolute ethanol as was also prenylamine. The other drugs used were dissolved in deionized and twice redistilled water. After incubation the cells were spun down at 2000×*g* for 5 min and were then resuspended in two changes of fresh incubation medium to wash out any adhering reserpine. Isolated mast cell granules were incubated in 0.30 M sucrose pH 6.9, spun down at 2700×*g* for 30 min and washed once by resuspension in fresh sucrose. The washed cell pellet was lysed in 0.5—1.0 ml of 0.1 N HCl. The washed granules were resuspended in the same volume of sucrose which was acidified to pH 1.0 with HCl.

Determination of reserpine. The reserpine content of the lysed pellets was determined according to Jakovlevic *et al.* (1962). Reserpine was extracted into 3.0 ml of chloroform by shaking for 30 min in 12 ml teflon stoppered pyrex test tubes. After centrifugation [illegible]00 μl of the chloroform layer was added to 900 μl of a 1 [illegible] p-toluene sulfonic acid solution in glacial acetic acid in 3 ml selected pyrex tubes. After vigorous mixing the reaction was completed by heating at 95—100 °C for 30 min and after cooling to room temperature the fluorescence was read at 480 nm after activation at 380 nm (uncorrected instrumental readings) using an Aminco-Bowman spectrofotofluorometer or a *Farrand Ratio Fluorometer* furnished with a Corning 7—60 glass filter as primary filter and with Corning 3—72 and 5—61 glass filters as secondary filter. The method has previously been used to determine the reserpine content in pharmaceutical preparations and was reported to be linear in the range 0.005 to 5.0 μg reserpine per ml (Jakovlevic *et al.* 1962). We were able to confirm this linearity using biological material. The recovery of the method proved satisfactory using biological material [illegible] 96±2% (mean and S.E.) of the calculated amount was recovered from the internal standard. Using isolated mast cells low tissue blank readings corresponding to 0.00[illegible] μg reserpine were obtained. Cellular material resuspended in sucrose gave somewhat higher blank readings. The variation coefficient of the method was below [illegible]. Reserpine base (Sigma Chemical Company) dissolved in glacial acetic acid was used as standard. The method has been reported not to measure oxidative breakdown products of reserpine (Jakovlevic *et al.* 1962). The possible interference of biological metabolites was not evaluated. None of the drugs used interfered with the reserpine fluorescence.

Determination of 5-HT. The 5-HT content of the aqueous phase after extraction with chloroform was determined fluorometrically after extraction into n-butanol as described by

TABLE I Effect of albumin concentration in the incubation medium on the incorporation of reserpine into isolated mast cells in vitro

	Incorporation of reserpine nmol/10 mast cells
No albumin (control)	0 445 ± 0 013 (100 0)
0 1 albumin	0 381 ± 0 078 (85 6)
1 0 albumin	0 181 ± 0 013 (40 6)

Incubation with reserpine at 5×10^{-6} M for 60 min at 37 C. Figures in brackets correspond to the uptake of reserpine in per cent of control uptake Means and S E of 1 expt in triplicate

Weissbach (1961) using an Aminco-Bowman spectrophotofluorometer 5 HT standards (5 hydroxytryptamine creatinine sulphate Fluka AG) were present during the chloroform extraction. No loss of 5-HT during this extraction occurred The 5 HT and reserpine contents of the isolated mast cells were expressed as nmoles per 10^6 mast cells and refer to the bases Mast cells were counted in the Burker chamber

Reagents Deionized and twice redistilled water was used throughout The reagents used in the analyses were commercially available analytical grade products p-Toluene sulfonic acid (Merck AG) was stored desiccated and made up freshly for every determination n butanol and n-heptane were washed according to Weissbach (1961)

Drugs Reserpine (Sigma) 5 hydroxytryptamine creatinine sulphate and 5 hydroxytryptophan (Fluka AG) prenylamine (Segontin® lactate Hoechst AG) imipramine HCl (Medica Ltd) tetrabenazine (Nitoman substance Roche Ltd) dl amphetamine sulphate (Siegfrieds AG) FCCP carbonyl cyanide p-trifluoromethoxy phenylhydrazone was obtained from Dr M Wikstrom Department of Clinical Chemistry University of Helsinki as a sample of a gift from P G Heytler The concentrations of the drugs refer to the salts with the exception of those of reserpine and 5 HT which refer to the bases

Results

The incorporation of reserpine into isolated mast cells as reported earlier (Jansson Gripenberg and Partanen 1971 Gripenberg and Jansson 1971) was confirmed At an exogenous concentration of 5×10^{-6} M 0 372 ± 0 039 nmol reserpine per 10^6 isolated mast cells was incorporated in 1 h at 37° C (mean ± S E of 21 expts in triplicates) Assuming that the volume of 10^6 mast cells is 1 µl this amount gives a concentration ratio of 75 between intracellular reserpine and reserpine in the medium This ratio was not changed much by incubation in reserpine concentrations from 10^{-6} to 10^{-5} M

Effect of albumin on the incorporation of reserpine

Reserpine apparently becomes bound to albumin as reported earlier (Solomon and Zieve 1967) With albumin present in the medium the incorporation of reserpine into mast cells was reduced (Table I) However albumin at 0 1 % did not greatly decrease the incorporation in comparison with the incorporation with no albumin present Thus albumin at 0 1 % was routinely added to the incubation medium as the presence of small amounts of albumin or serum has been reported to be essential for the preservation of isolated mast cells (Uvnas and Thon 1959 1961) No a was made to find out whether reserpine might form precipitates with other c uents in the medium but in Table II the results of two experiments in w h

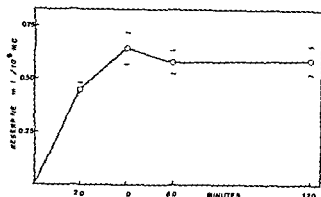


Fig 1 Incorporation of reserpine into mast cells as a function of time Incubation with reserpine at 5×10⁻⁶ M at 37 C Results of 2 expts incubated and determined in duplicates or triplicates

amount of precipitable or "inactive" reserpine was evaluated are presented It is evident that about 5 % of the total amount of reserpine at 5×10^{-6} M could be spun down at 80 000 gmin The reserpine concentrations in the present paper are not corrected for this precipatable amount

Incorporation of reserpine as a function of time

Reserpine was rapidly incorporated into isolated mast cells At an exogenous concentration of 5×10^{-6} M a saturated level was achieved in less than 40 min (Fig 1) Incubation periods of less than 20 min in length were not tried

Incorporation of reserpine as a function of concentration

Barely detectable amounts of reserpine with the method used were incorporated below or at an exogenous concentration of 10^{-7} M (Fig 2) The incorporation at 10^{-6} M was still low Higher concentrations accelerated the incorporation with no tendency to saturation at the concentrations tested

TABLE II Evaluation of the amount of reserpine precipitated upon incubation in a modified Krebs Ringer solution

Reserpine dissolved in	Absolute ethanol	0 25 glacial acetic acid in absolute ethanol
Reserpine recovered after centrifugation nmoles		
– Bottom layer	3 89 (76)	2 63 (52)
– Top layer	1 18 (24)	2 37 (48)
Difference – Precipitable amount of reserpine nmoles	2 (4 53)	0 26 (5)

Both reserpine stocks held 5 10⁻ M Reserpine was added at 5 10 M to 1 0 ml of the modified Krebs-Ringer solution and incubated for 60 min at room temperature The tubes were then centrifugated for 30 min at 2 00 g Exactly half the volume was carefully pipetted off (= TOP LAYER) and the reserpine content of this volume and the remaining volume at the bottom of the tubes (= BOTTOM LAYER) was determined The figures in the brackets correspond to the amount of reserpine in per cent of total added Mean of 2 expts in triplicates

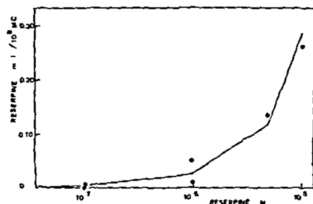


Fig 2 Incorporation of reserpine into isolated mast cells as a function of the extracellular reserpine concentration Incubation for 1 h at 37 C Results of 1 expt in duplicate

Incorporation of reserpine as a function of temperature

The incorporation of reserpine into mast cells was temperature dependent In the experiments presented in Table III about the same amount of reserpine was incorporated at 0 23 and 37° C respectively

Incorporation of reserpine as a function of pH

A maximal incorporation of reserpine into mast cells was obtained at pH 7 5—7 7 An increase of pH to 8 2 decreased the incorporation by some 20 % At pH 7 0 which was used routinely in the present paper the incorporation was decreased by some 20 % and further lowering of the pH to 6 6 caused an almost 50 % drop of the incorporation (Fig 3)

Effect of drugs on the incorporation of reserpine

Blockers of amine uptake like imipramine and prenylamine were without effect on the incorporation at 10^{-5} M (Table IV) Metabolic inhibitors NaCN at 10^{-4} and 10^{-3} M and the potent uncoupler of oxidative phosporylation FCCP at 3×10^{-6} M were also without significant effect on the incorporation of reserpine (Table IV) 5 HT 5 hydroxytryptophan tetrabenazine and dl amphetamine ere likewise without effect in this respect (Table IV)

TABLE III Incorporation of reserp n into i olat d mast cells as a function of temperatur

Tempe ature C	Incorpo at on of eserp ne nmol/10 mast cells
0	0 685 ± 0 008
23	0 656 ± 0 026
37	0 665 ± 0 009

Incubation with eserpine at $5\times10^{-}$ M for 60 m n Means and S E of 1 expt in

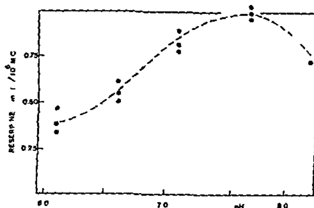


Fig 3 Effect of pH on the incorporation of reserpine into isolated mast cells Incubation with reserpine at 5×10^{-6} M for 1 h at 37 C The cells were collected and isolated as usual but thereafter resuspended and incubated in 0 15 M phosphate buffer of the appropriate pH Results of 1 expt in triplicate

Storage of reserpine in mast cells

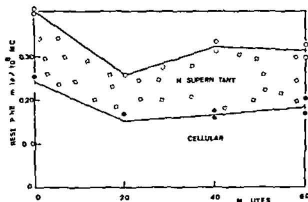
Differential centrifugation of a mast cell population pre loaded with reserpine and degranulated by freezing and thawing produced a practically pure mast cell granule fraction which held 23 % of the total amount of reserpine recovered (Table V) In trying to obtain a granule fraction richer in reserpine several degranulation methods were employed homogenisation with an Ultra Turrax homogenisation device homogenisation with a Potter Elvehjem ground glass homogenizer homogenisation with a smooth glass plexiglass homogenizer with a 250 μm gap degranula

TABLE IV *Effect of drugs on the incorporation of reserpine into isolated mast cells*

Drug added	Concentration	Incorporation of reserpine nmol/10⁶ mast cells	Number of experiments
None (control)		0 557 ± 0 066	2
Imipramine	10⁻ M	0 524 ± 0 077	2
Prenylamine	10 M	0 484 ± 0 053	2
None (control)		0 430 ± 0 023	2
NaCN	10 M	0 470 ± 0 043	2
None (control)		0 489 ± 0 092	2
NaCN	10 M	0 479 ± 0 108	2
None (control)		0 401 ± 0 036	1
FCCP	3 10 M	0 390 ± 0 002	1
None (control)		0 463 ± 0 016	2
5 HT	2 5 10 M	0 478 ± 0 046	2
None (control)		0 475 ± 0 036	1
5 HTP	4 5 10 M	0 365 ± 0 023	1
None (control)		0 560 ± 0 016	2
Tetrabenazine	10 M	0 501 ± 0 031	2
dl amphetamine	10 M	0 494 ± 0 024	2

Pre incubation with the drugs for 30 min (5 HTP for 60 min) Further incubation with reserpine at 5 10⁻⁶ M for 60 min at 37 C Means and S E of 1 – 2 expts each one made in triplicate

Fig 4 Storage of reserpine in isolated mast cells in vitro The cells were pre incubated with reserpine at 5×10^{-6} M for 1 h at 37 C The cells were then washed twice and resuspended and further incubated in fresh Krebs-Ringer solution at 37 C After the incubation the cells were spun down at $8000 \times g$ for 5 min in a refrigated centrifuge Results of 1 expt in duplicate



tion by osmotic shock (0 03—0 096 M sucrose) and by prolonged incubation in isotonic sucrose (ad 24 h on ice water) None of the homogenisation procedures was superior to the ordinary freeze thawing which was used routinely Degranulation by osmotic shock or by prolonged incubation in sucrose produced a granule fraction somewhat richer in reserpine than after freeze thawing but the results were not easily *reproducible* The results given in Table V are therefore obtained after freezing and thawing and show that the granular sediment holds 23 % of the total amount of reserpine while the heavy sediment and the final supernatant hold 40 % and 35 % respectively When cells which were incubated with reserpine and washed 2 times were resuspended in fresh incubation medium it was observed that already at zero time more than one third of the total amount of reserpine recovered was found in the supernatant The same amounts were found in the supernatants after different periods of reincubation (Fig 4) The cell bound reserpine was decreased by some 35 % after reincubation for 1 h at 37° C In another experiment the reincubation in fresh medium was continued for 2 h with the same result roughly one third of the total amount of reserpine in the supernatant This con

TABLE V Compartmentati n of reserpin and 5 HT in isolated mast cells

Fraction	Reserp ne of total	5 HT of total
Sed m $350 \times g$	40±4 (1 48)	30±8 (14 0)
Sed m ? $700 \times g$	23±5 (1 12)	25±5 (9 82)
Sup rn tant	35±4 (1 26)	47±6 (18 3)

Incubation w th reserpine at 10 M for 30– 60 min at room tempe ature After epeated washing in Krebs R nger olution and sucro e th cells were reu uspended in 0 30 M sucrose pH 6 9 and frozen and th wed 3 t mes The figures give rese p ne and 5 HT n the fr tions obtained after d fferential centrifugat on in p r cent of total amount re overed F gu es in brackets give correspond ng amounts n nmoles As th e figures d pend on the amount of cellula used they var ed f om one experiment to another In calculating the means in ed-off figu es we us d and thus they do not exactly correspond to the amounts given Means (nmoles) or means and S E of 5 (5-HT) or 6 (r serp ne) expts

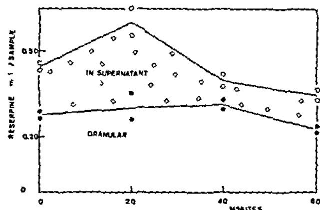


Fig 5 Storage of reserpine in isolated membrane free mast cell granules The granules were obtained by differential centrifugation after freezing and thawing of a mast cell population in sucrose Incubation with reserpine at 10 M for 30 min at room temperature The granules were then washed once with 0.30 M sucrose pH 6 9 and resuspended and further incubated in fresh sucrose After incubation the granules were spun down at 8000 ×g for 10 min in a refrigerated centrifuge Results of 1 expt in duplicate

siderable loss of reserpine could not be explained by poor washing of the cells because 3 washes do not further lower the reserpine content in comparison with the content of reserpine after 2 washes (own observations)

In checking the possibility that this easily removable reserpine fraction might be located in the clear cytoplasm the same experiment as above was repeated with isolated mast cell granules (Fig 5) The same result as with intact mast cells was obtained At zero time about 40 % of the total amount of reserpine was found in the supernatant After an incubation of 20 min the granule bound reserpine was at the same level as at zero time but surprisingly high amounts were recovered from the supernatant This was probably due to an experimental artefact because after 40 and 60 min of incubation the amounts of reserpine in the supernatant were of the same magnitude as at zero time The granule bound reserpine decreased by some 20 % after 1 h of reincubation

Incorporation of reserpine into isolated mast cell granules

Reserpine was rapidly incorporated into isolated membrane free mast cell granules obtained after freezing and thawing As for intact mast cells the uptake was rapid

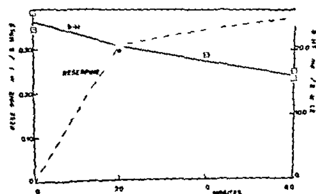


Fig 6 Incorporation of reserpine into isolated membrane free mast cell granules The granules were obtained by differential centrifugation after freezing and thawing of a mast cell population in sucrose Incubation for different periods of time at room temperature with reserpine at 10 M and 5 HT at 7.5×10^{4} M added simultaneously Results of 1 expt in duplicate

during the first 20 min reaching an almost saturated level while the uptake during the succeeding 40 min was small (Fig 6) The presence of 5 HT in high concentrations in the medium (2.5×10^{-5} M) did not affect the incorporation of reserpine The uptake of 5 HT was on the other hand completely blocked and the endogenous 5-HT level was even somewhat lower after incubation than at zero time

Discussion

According to the present study the incorporation of reserpine into isolated mast cells shows the following features The incorporation is rapid reaching a maximum level in less than 40 min However the incorporation increased in proportion to increasing exogenous concentration with no tendency to saturation at the range tested The incorporation was not temperature dependent nor could it be blocked by metabolic inhibitors These results are taken to suggest that reserpine is incorporated into mast cells by a passive mechanism, probably by its lipid solubility The results obtained support earlier observations on this subject (Solomon and Zieve 1967 Wagner and Stitzel 1969)

Reserpine was accumulated against a concentration gradient until concentration ratios (mast cells/incubation medium) of up to 75 1 were obtained Incorporation of reserpine into blood platelets has been reported to result in a concentration gradient of the same magnitude (Solomon and Zieve 1967) A passive incorporation against a concentration gradient is possible only if reserpine becomes bound to some intracellular storage organelle The binding capacity of this storage organelle seems to be rather high since the concentration ratio mast cells/incubation medium was not much changed at exogenous reserpine concentrations from 10^{-6} to 10^{-5} M It has been reported (Wagner and Stitzel 1969 Alpers and Shore 1969) that reserpine administered in vivo is more or less evenly distributed in all subcellular fractions of heart homogenates DaPrada and Pletscher (1969 a) on the other hand found that reserpine was recovered in the fraction holding the amine storing organelles of blood platelets In another work (DaPrada and Pletscher 1969 b) it was demonstrated that reserpine was bound to the membranes surrounding the amine storing organelles in the platelet

In the present work evidence was obtained showing that a considerable amount (about 40 %) of the total amount of reserpine incorporated is only loosely bound to the cells It can not be excluded that part of this easily removable reserpine is located in the clear cytoplasm The re incubation experiment with mast cell granules pre loaded with reserpine show however that about 40 % of the drug exists in a readily diffusible form This could be due to the physico-chemical properties of the intracellular storage organelle and of reserpine itself It has also been reported that after in vivo administration of reserpine some redistribution occurs during homogenisation and centrifugation processes (Alphers and Shore 1969 Wagner & Stitzel 1969)

The $2700\times g$ sediment (granule sediment) holds 1 12 nmol reserpine (Table V) which corresponds to 43 % of the total amount of particle bound reserpine The $350\times g$ sediment thus holds the remaining 57 % of the particle bound drug The corresponding figures for 5 HT in both sediments are 41 % and 59 % respectively This very close correlation between endogenous particle bound 5 HT and incorporated particle bound reserpine suggests that reserpine to a major part becomes bound to the amine storing mast cell granules This suggestion is further supported by the fact that reserpine is readily incorporated into isolated membrane free mast cell granules

Taking the result of a large material 3 26 nmol 5 HT is taken up in 1 h at 37 C by 10^6 mast cells at an exogenous concentration of $2\,5\times10^{-6}$ M (Jansson 1970 a) Reserpine at 10^{-5} M inhibits some 85 % of this uptake which corresponds to 2 75 nmol 5 HT/10^6 mast cells The incorporated amounts of reserpine is about 0 430 nmol/10^6 mast cells under these conditions because 5 HT was not able to inhibit the incorporation of reserpine (present results) The reserpine blocked uptake of 5 HT thus corresponds to 2 75 nmol 5 HT/10^6 mast cells and the incorporated amounts of reserpine correspond to 0 430 nmol reserpine/10^6 mast cells It can therefore be concluded that one molecule of reserpine blocks the uptake of 6 molecules of 5 HT by mast cells in vitro

The mechanism of this reserpine blocked uptake of 5 HT into mast cells is propably different from the mechanism which is responsible for the reserpine induced release of 5 HT from platelets in vitro since it has been reported that in this model one molecule of reserpine affects the release of several hundreds of 5 HT molecules (Carlsson Shore and Brodie 1957)

In conclusion the present results indicate that in mast cells there is a close correlation between the incorporation of reserpine and the drug induced inhibition of uptake of 5 HT by these cells in vitro The results support earlier findings according to which incorporated reserpine becomes bound to the amine storing organelles in platelets (DaPrada and Pletscher 1969 a b) and furthermore they also support an earlier suggestion (Jansson 1970 b) that in mast cells reserpine has a granular mechanism of action Experiments designed to test these results in vivo and on simple neuronal models are in progress

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Acta physiol scand 1972 86 444—455

From the University Laboratory of Physiology and the Radcliffe Infirmary Oxford

Comparison of the Effect of Different Types of Exercise on the Baroreflex Regulation of Heart Rate

By

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Abstract

Cunningham D J C E S Petersen R Peto T G Pickering and P Sleight
Comparison of the effect of different types of exercise on the baroreflex regulation of heart rate Acta physiol scand 1972 *86* 444—455

The reflex cardiac slowing following a transient druginduced rise of arterial pressure was studied in ten subjects at rest and during three different kinds of exercise bicycling with the legs sustained isometric hand grip and rhythmic isometric hand grip All three kinds resulted in a variably diminished reflex response When compared at the same heart rate the reflex sensitivity was significantly more depressed during sustained hand grip than during bicycling the response during rhythmic hand grip was not significantly less than during bicycling The changes in the reflex response were unrelated to the oxygen consumption which increased markedly during pedalling but very little during sustained hand grip Total circulatory occlusion to the exercising arm did not alter the change in the reflex response during sustained hand grip Maintaining the occlusion after the exercise had ceased prevented the arterial pressure but not the heart rate from falling to its resting level The reflex response recovered to 50 of its resting value The reflex sensitivity is correlated well with the prevailing arterial pressure (Po) better with the prevailing pulse interval (Io) and best with both together (Io/Io) It is not certain whether the pressure and pulse interval directly influence reflex sensitivity or vice versa or whether some other influence is changing Io Po and reflex sensitivity in parallel

During dynamic bicycling exercise in man the reflex bradycardia in response to a drug induced rise of arterial pressure declines progressively as the work load is increased such that at a heart rate of 150/min or more the reflex bradycardia is virtually abolished (Bristow *et al* 1971) The change in the reflex response appeared to be closely related to the intensity of the work For this kind of exercise work intensity heart rate and oxygen consumption are all linked and it was therefore not possible to decide which relation was the more fundamental *i e* between the reflex response and other circulatory changes on the one hand or between the

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reflex response and the more general need to transport oxygen as measured by the oxygen consumption on the other

Sustained hand grip differs from bicycling exercise in several respects Oxygen consumption changes very little despite the occurrence of a considerable tachycardia but the most striking circulatory change is a large rise of arterial pressure (Lind *et al* 1964) The present experiments were undertaken to answer two questions first does the decline in the reflex response follow the circulatory or the metabolic changes? Secondly is the reflex affected in the same way by a kind of exercise which primarily changes arterial pressure (hand grip) as by one which primarily changes heart rate (bicycling)?

Methods

10 healthy subjects aged 19 to 30 years were studied Arterial pressure was recorded from a thin polythene cannula whose tip lay in the axillary artery A second cannula was put in a superficial vein sudden injections of the α adrenergic drug phenylephrine (Boots Pure Drug Co Ltd.) in doses of 50—150 μg were made through this to produce a transient rise of arterial pressure of about 20 mm Hg The effect of such injections is very brief and they could be repeated at three minute intervals The arterial pressure was recorded on a slow paper trace (2.5 mm/s) and also on magnetic tape (19 mm/s) The baroreflex response was evaluated by a method described previously (Smyth Sleight and Pickering 1969 Bristow *et al* 1971) following an injection of phenylephrine pulse interval is related to the preceding systolic pressure on a beat to beat basis starting from the end of the injection and proceeding to the peak systolic pressure This relation is effectively linear and the slope (regression coefficient) of the regression line of pulse interval on systolic pressure expressed as ms/mm Hg is taken as an index of reflex sensitivity The calculations of reflex sensitivity were done by playing the tape through an analog to-digital converter into an IBM 1130 computer The computer measured the mean pressure of each beat as well as the systolic pressure and reflex sensitivity could be expressed using either measure of arterial pressure as the stimulus and pulse interval as the response As found previously (Bristow *et al* 1971) the values of reflex sensitivity were similar to the two Except where stated otherwise systolic pressure has been used in this paper for the calculations of reflex sensitivity

All the experiments were done with the subjects sitting on a bicycle ergometer (Jaeger Wurzburg) For the first 5 expts. the subjects breathed pure oxygen (from a spirometer in a closed circuit for estimation of oxygen uptake In later experiments end tidal PCO_2 was held constant at 38—40 torr

Three different types of exercise were used and the subjects were rehearsed in all of them before the experiment. The first was pedalling the bicycle ergometer at 2 or 3 different work loads from 30 to 85 W Phenylephrine injections were made at 3 min intervals 3 at each work load) the first injections being given not less than 4 min after the start of each work load The second was sustained isometric hand grip using a strain gauge dynamometer of the type described by Clark Hellon and Lind (1958) The strain was recorded on the paper trace together with arterial pressure A graduated dial was placed facing the subject and this displayed the strain so that he could maintain a constant grip of the right intensity At the start of each experiment the subject's maximum voluntary contraction (MVC) was determined and subsequent handgrip were done at 30 MVC Hand grip was maintained for 3 min and one phenylephrine injection was made towards the end of this period Hand grips were also performed during total circulatory occlusion of the arm produced by inflating a sphygmomanometer cuff on the upper arm to a pressure of 250 mm Hg This occlusion was started 3 min before the hand grip and kept on for 3 min after for a total period of 9 min. One phenylephrine injection was made during the period of occlusion before the grip one during the grip and one during the occlusion after the grip The third type of exercise was rhythmic hand grip (30 45 and 60 % MVC) timed by a metronome beating once per second (grip for 1 s rest for 1 s grip for 1 s etc) Two or more phenylephrine injections were given during this time

The order of the different types of exercise was varied in different experiments, there were rest periods in between during which further injections were made

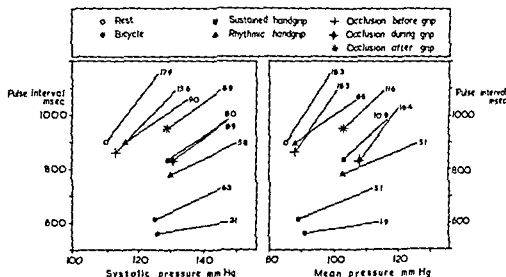


Fig 1 Each line is the average response to 2 or more phenylephrine injections in one experimental situation in one subject (T C) The position of each symbol at the base of a line represents the pre-injection values of arterial pressure and pulse interval The numbers by each line refer to the reflex sensitivity for that situation Left hand panel reflex sensitivity calculated in terms of systolic pressure right hand panel in terms of mean pressure The two work loads for pedalling are shown separately

Results

Relation between circulatory and metabolic changes

All 3 types of exercise were associated with a rise of arterial pressure and a decrease of pulse interval in all 3 there was also a decline of reflex sensitivity The phenylephrine regression lines are shown for one subject in Fig 1, expressed in terms of both systolic and mean pressures The data for the individual subjects are given in Table 1 Oxygen consumption rose markedly with bicycling exercise but neither sustained nor rhythmic hand grip increased it by much despite the large circulatory changes This is illustrated in the left hand panel of Fig 2 where the oxygen consumption is plotted against the pre injection pulse interval for different types of exercise in one subject The right hand panel of the figure shows that reflex sensitivity fell during both types of hand grip even though oxygen consumption showed almost no change It is thus clear that the changes of reflex sensitivity during exercise follow the other circulatory changes rather than the changes in overall metabolic rate

Comparison of hand grip with bicycling

(a) *Sustained hand grip* The fastest heart rates (average 117/min pulse interval 513 ms) were seen with the highest load of bicycling mean pressure rose by only 10 and systolic by 24 mm Hg Sustained hand grip produced less tachycardia (average rate 90/min pulse interval 665 ms) the rise of mean pressure (20 mm Hg) but

TABLE I Changes in blood pressure, heart rate, oxygen consumption and reflex sensitivity during different kinds of exercise

I_0 = pre-injection pulse interval (msec)
P_0 = pre-injection systolic pressure (mm Hg)
Slope = baroreflex sensitivity (msec/mm Hg)
$\dot{V}O_2$ = oxygen consumption (ml/min)
MAP = pre-injection mean pressure (mm Hg)

Subject	Condition	I_0	MAP	P_0	I_0/P_0	Slope	$\dot{V}O_2$
J.L.	Rest	878	79	110	7.52	9.7	298
	Pedalling 30 W	607	86	124	4.89	6.4	912
	– 80 W	455	91	133	3.42	0.6	1074
	Grip 30% MVC	631	107	139	4.54	6.3	337
	Occlusion before grip	872	87	114	7.65	8.7	
	– during –	674	101	133	5.07	5.3	300
	– after –	872	105	140	6.23	8.3	
	Rhythmic grip 30%	706	86	119	5.93	10.4	–
	– – 45%	674	97	131	4.76	12.5	382
	– – 60	577	104	140	4.12	3.6	–
L.C.	Rest	784	77	108	7.26	13.8	–
	Pedalling 30 W	619	84	121	5.16	6.5	–
	– 70 W	522	95	131	3.98	1.9	–
	Grip 30 MVC	618	106	136	4.51	3.7	–
	Occlusion before grip	765	75	107	7.15	14.7	–
	– during –	650	107	132	4.97	9.5	–
	– after –	992	102	133	7.46	9.0	–
	Rhythmic grip 30	670	91	121	5.54	8.1	–
T.C.	Rest	897	85	110	8.15	17.9	–
	Pedalling 50 W	612	89	125	4.90	6.3	–
	– 85 W	560	91	126	4.44	7.1	–
	Grip 30 MVC	838	103	129	6.50	8.0	–
	Occlusion before grip	866	88	113	7.66	13.6	–
	– during –	835	108	131	6.37	8.9	–
	– after –	951	103	129	7.37	8.9	–
	Rhythmic grip 30	899	88	116	7.75	9.0	–
	– – 45	850	98	123	6.91	8.9	–
	– – 60	779	103	130	5.99	5.8	–
I.A.	Rest	680	81	117	5.81	11.4	393
	Pedalling 15 W	516	89	133	3.88	6.7	–
	– 30 W	508	80	126	4.03	4.4	1200
	– 60 W	502	80	129	3.89	3.3	1430
	Grip 30 MVC	580	103	139	4.17	5.0	543
D.S.	Rest	779	93	122	6.38	17.5	299
	Pedalling 25 W	569	100	139	4.09	7.7	930
	– 60 W	500	95	140	3.57	4.9	1100
	Grip 30 MVC	600	119	154	3.90	6.4	409
	Occlusion before grip	808	96	123	6.60	10.2	
	– during –	605	117	158	3.87	9.6	–
	– after –	825	108	149	5.54	9.9	
	Rhythmic grip 30	655	114	146	4.49	6.4	530
S.W.	Rest	934	79	114	8.19	27.1	323
	Pedalling 50 W	597	83	128	4.66	5.0	1147
	– 80 W	483	86	137	3.52	2.6	1575
	Grip 30% MVC	787	100	132	5.96	8.2	360
	Occlusion before grip	805	82	117	6.88	26.0	–
	– during –	659	97	133	4.95	6.0	458
	– after –	885	88	125	7.08	15.6	–

Subject	Condition	I_0	MAP	P_0	I_0/P_0	Slope	VO
	Rhythmic grip 30 %	918	85	122	7 52	12 0	390
	— — 45 %	841	89	125	6 73	11 0	38?
	— — 60 %	682	89	130	5 25	10 0	450
J S	Rest	698	84	114	6 12	13 9	—
	Pedalling 50 W	541	100	160	3 38	4 1	—
	— 80 W	480	107	169	2 84	1 3	—
	Grip 30 % MVC	607	89	144	4 21	7 0	—
	Occlusion before grip	772	91	122	6 33	13 7	—
	— during —	590	102	136	4 34	8 7	—
	— after —	657	105	138	4 76	10 6	—
	Rhythmic grip 30	629	89	124	5 07	10 1	—
	— — 45 %	598	104	140	4 27	4 3	—
	— — 60	628	119	155	4 05	4 9	—
R L	Rest	799	93	121	6 60	17 2	—
	Pedalling 35 W	616	106	143	4 31	10 0	—
	— 70 W	553	107	138	4 01	9 ?	—
	Grip 30 % MVC	580	124	15?	3 81	2 4	—
	Occlusion before grip	773	92	122	6 33	13 7	—
	— during —	596	121	148	4 03	4 0	—
	— after —	850	116	144	5 90	16 6	—
	Rhythmic grip 30	696	112	140	4 97	14 0	—
	— — 40 %	550	107	141	3 90	2 6	—
A P	Rest	69?	85	117	5 91	23 4	—
	Pedalling 20 W	522	88	132	3 95	9 2	—
	— 35 W	512	93	137	3 74	7 2	—
	Grip 30 MVC	6?3	100	130	4 79	8 9	—
	Occlusion before grip	713	87	119	5 99	18 6	—
	— during —	670	119	155	4 32	8 3	—
	— after —	749	103	1?5	5 99	21 0	—
	Rhythmic grip 20	687	93	124	5 54	14 1	—
	— — 30	684	97	127	5 38	14 6	—
P R	Rest	88?	85	119	7 44	24 3	—
	Pedalling 30 W	655	96	141	4 64	4 6	—
	60 W	558	100	149	3 75	6 5	—
	Grip 30 MVC	791	8?	129	6 13	13 9	—

not systolic (24) was greater The average changes for all the subjects are shown in Fig 3 and the individual data in Table I

The changes in the reflex response are shown for one subject in Fig 1 the left and right hand panels show the changes plotted against systolic and mean pressure respectively The values of reflex sensitivity when calculated for systolic and mean pressure are similar and show similar changes with exercise The values for reflex sensitivity that follow refer to systolic pressure

Bicycling not only produced the greatest tachycardia but also the greatest depression of reflex sensitivity We found previously (Bristow *et al* 1971) that when for bicycling exercise at several different work loads the slope of each phenylephrine line is plotted against the preinjection pulse interval the relation is seen to be roughly linear with an intercept corresponding to zero slope at a pulse interval of about

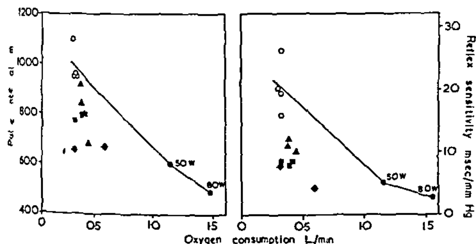


Fig 2. Results for subject S W symbols as for Fig 1 In the left hand panel the oxygen consumption for different types of exercise is plotted against the pre injection pulse interval and in the right hand panel against reflex sensitivity The lines join the points for rest and pedalling exercise the points for the other kinds of exercise do not lie on these lines

400 ms (heart rate 150/min) Such a plot of the present data is illustrated in Fig 3 (top right panel) The point for sustained hand grip lies below the line joining the points for rest and the two bicycling work loads i e when compared at the same heart rate reflex sensitivity is lower during sustained hand grip than during bicycling From the linear regression relating slope and the pre injection pulse interval during bicycling we predicted slope values for the pulse interval occurring during hand grip These predicted values were significantly ($p < 0.001$) lower than the observed values for hand grip (t test for paired data)

One possible explanation for this difference might be that when phenylephrine is injected towards the end of a sustained hand grip arterial pressure and pulse interval have not yet stopped rising and falling respectively and hence produce a spuriously low value for reflex sensitivity The method used to test this possibility was to measure the rate at which pressure and pulse interval were changing for the 15 s preceding the phenylephrine injection neither rate differend significantly from zero (15 s being approximately the time over which the phenylephrine regressions are calculated) These findings mean that the phenylephrine slopes for sustained hand grip are unlikely to be spuriously low because the circulatory changes due to the drug are so much quicker than those due to the hand grip

(b) *Rhythmic hand grip* The average changes of arterial pressure and heart rate during rhythmic grip were intermediate between those during bicycling and sustained grip i e at the same heart rate the pressure was usually higher than during bicycling but not as high as during sustained grip (but there was considerable overlap and neither difference was significant) Reflex sensitivity was also decreased by rhythmic grip again the average changes were intermediate between those

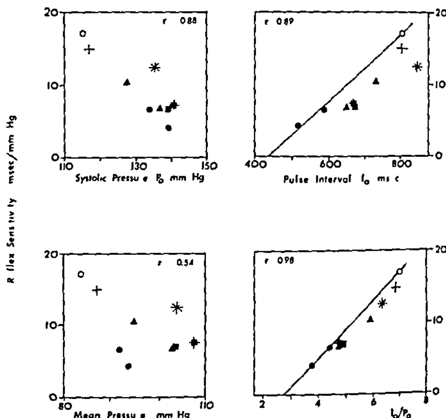


FIG 3 Average results of all the subjects symbols as for Fig 1 Reading from top left to bottom right reflex sensitivity is plotted against (a) systolic pressure (I_o) (b) pulse interval (I) (c) mean pressure and (d) I/I r = correlation coefficient for each relation

seen during bicycling and static hand grip but were significantly different from neither (Fig 3)

(c) *Circulatory occlusion* Alam and Smirk (1938) and later Donald *et al* (1967) found that when exercise is performed with the circulation to the exercising arm occluded the arterial pressure remains high after the exercise has stopped although heart rate returns quickly to resting levels This was of particular interest in the present experiments as it means that we can test the reflex at a time when the arterial pressure is raised but the heart rate is near normal Occlusion was maintained for nine min with a 3 min sustained hand grip (30 % MVC) in the middle of this period One phenylephrine injection was given before the grip one during and one after Typical results are shown in Fig 1 and 3 Occlusion without exercise had little effect on either arterial pressure or heart rate and reflex sensitivity while tending to be lower was not significantly changed Hand grip during occlusion produced an average rise of mean pressure of 28 mm Hg systolic of 26 and a fall of pulse interval to 660 ms (rate 91/min) These changes were slightly but not

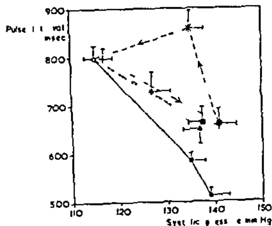


Fig 4 Mean values of steady state pulse interval I_0 plotted against mean systolic pressure P for all types of experimental situations Symbols as for Fig 1 Bars indicate SEM Note marked changes with all types of exercise in both I_0 and P_0 with occlusion during grip a further pressure rise is seen with occlusion after grip pressure remains high while pulse interval returns to or above resting level

significantly larger than those due to grip without occlusion The depression of reflex sensitivity was also the same as for grip without occlusion (Fig 3 and 4) When the grip was released the pulse interval returned promptly to its resting value or in some cases above it whereas the mean pressure fell by only 4 mm Hg on average being still 24 mm above its resting level During this period reflex sensitivity returned half way towards the resting level (Fig 1 and 3)

The subjects nearly always admitted to discomfort towards the end of the grip and during the sub equent rest with occlusion but most of them denied that the discomfort was the same as pain

Relation between reflex sensitivity and other circulatory variables

The data from our previous experiments on bicycling exercise (Bristow *et al* 1971) suggested that the changes of reflex sensitivity were more closely linked to the changes of pulse interval than of systolic pressure which were small While the present data covering a wider range of conditions support this view in general (Fig 4) there are notable exceptions for example during circulatory occlusion after the end of a sustained hand grip the pulse interval returns to its resting value but reflex sensitivity only recovers by 50 % One possible interpretation of this is that reflex sensitivity is a function of arterial pressure as well as of pulse interval although it must be remembered that it is not known which is the dependent and which the independent variable in this case (see Discussion)

In order to examine the relation between the three sets of variables in more detail the method of analysis of variance was used The mean reflex sensitivity for all the data was 9 7 ms/mm Hg with a range from 0 6 to 24 3 and a total variance of 1360 Table II shows how the residual variances could be reduced by relating reflex sensitivity to either pulse interval (I) or systolic pressure (P) alone or in combination. The data were also examined for differences between individual subjects and

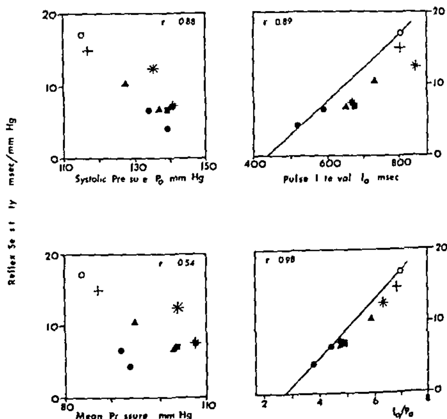


Fig 3 Average results of all the subjects symbols as for Fig 1 Reading from top left to bottom right reflex sensitivity is plotted against (a) systolic pressure (P) (b) pulse interval (I) (c) mean pressure and (d) I /P_o r = correlation coefficient for each relation

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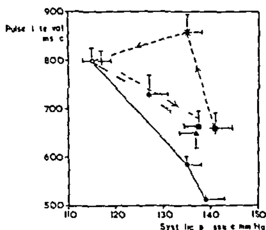


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In order to examine the relation between the three sets of variables in more detail the method of analysis of variance was used The mean reflex sensitivity for all the data was 9 7 ms/mm Hg with a range from 0 6 to 24 3 and a total variance of 23 66 Table II shows how the residual variances could be reduced by relating reflex sensitivity to either pulse interval (I) or systolic pressure (P) alone or in combination. The data were also examined for differences between individual subjects and

TABLE II Reductions in the sum of squared errors by predicting reflex sensitivity from other parameters

Parameters adjusted	Nil (0)	P_0 (1)	I_0 (1)	I_0/P_0 (1)	I_0/P_0+P_0 (2)	I_0/I_0+I_0 (2)	I_0+P_0 (2)
Nil (0)	—	722	896	1085	1095	1095	1085
Individual subjects (9)	393	1361	1436	1734	1770	1770	1734
Subjects + experimental situations (17)	1815	1855	1829	1851	1861	1863	1802
Total sum of squared errors = 2366							

Numbers in parentheses = degrees of freedom (total 77)
I_0 = pulse interval
P_0 = systolic pressure

experimental situations Thus comparison of the first and second rows of Table II shows that allowing for individual differences of average reflex sensitivity gives a greater reduction of variance than when all the subjects are considered to be identical Inspection of the second row of Table II shows that if we allow for individual variation relating reflex sensitivity to pulse interval gives a slightly better fit (i e a greater reduction of variance) than to systolic pressure, but relating it to a combination of the two (as I /P) is much better than to either alone Other more complex equations relating reflex sensitivity to I /P_0 plus I_0 or P_0 gave only marginally better results Reflex sensitivity may be equally well related to the experimental situation (i e type of exercise) (third row of Table II) *But after allowing for both* experimental situation and individual variation there is still a correlation ($F_{1\,9}$ = 4 13 $p < 0\,05$) between reflex sensitivity and I /P

It is theoretically possible that reflex sensitivity determines the level of I and P though there is strong evidence against this (see later) to test this possibility multiple regressions were also computed the other way round i e with I and P as the dependent variables and reflex sensitivity as the independent Table III shows that the multiple regression of I /P on reflex sensitivity and experimental situation is

TABLE III Analysis of variance table for prediction of systolic pressure and pulse interval (as I_0/I_0) from reflex sensitivity and type of exercise

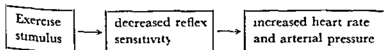
Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Reflex sensitivity	65 2	1	65 22	
Exercise type (after adjusting for reflex sensitivity)	30 8	8	3 85	$F_{8\,68}$ = 5 66 (P < 0 001)
Residual	46 2	68	0 68	
Total	142 2	77		

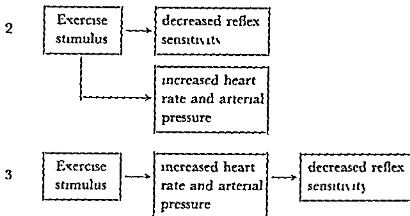
significantly better than that of I/P_0 on reflex sensitivity alone *i e* I_0/P cannot be accurately predicted from reflex sensitivity alone the experimental situation is also of importance

Discussion

All the kinds of exercise studied in these experiments that is sustained and rhythmic hand grip and pedalling with the legs were accompanied by a marked loss of baroreflex sensitivity They were also associated with variable increases in both heart rate and arterial pressure Previous workers have shown that oxygen consumption is affected little by sustained hand grip (Lind *et al* 1964) and our findings confirm this The oxygen consumption depends on the total work of contraction and thus to a major degree on the bulk of exercising muscle whereas the changes of arterial pressure and heart rate associated with sustained hand grip do not (Donald *et al* 1967) It is now clear that changes in baroreflex sensitivity are related more closely to the other circulatory changes than to the overall metabolic requirements of the body Reviewing the circulatory effects of sustained hand grip Lind *et al* (1964) were impressed by the large increase of arterial pressure compared with the small changes produced by bicycling they and Freyschuss (1970) suggested that such big changes could only occur if the baroreflex is suppressed during hand grip Their proposal is fully borne out by the present experiments At equivalent values of heart rate reflex sensitivity was significantly lower during sustained hand grip than during pedalling and the arterial pressure significantly higher The mean changes of heart rate and arterial pressure associated with rhythmic hand grip were intermediate between those found in leg and static arm exercise at equivalent values of heart rate reflex sensitivity tended to be lower than during pedalling but in this case the difference was not significant

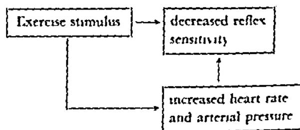
We found previously that situations in which there is an increase of heart rate (*e g* bicycling standing up and atropinisation) are associated with a fall of reflex sensitivity whereas slowing of the heart rate (*e g* sleep propranolol) are associated with an increase The present results are in general agreement with this (see Fig 4) with one notable exception occlusion after the hand grip In this situation the arterial pressure was raised but the heart rate had fallen to the resting level or a little below Reflex sensitivity was still depressed showing that the relation with heart rate is not invariable Two possible explanations for this depression of reflex sensitivity are (1) that it is a direct consequence of the high arterial pressure and (2) that there is a central depression of the reflex While it is clear from these and other experiments that exercise reflex sensitivity heart rate and arterial pressure are all closely related the causal sequence is not certain Three possibilities (not mutually exclusive) are shown below





The analysis of variance was used to investigate these relations in more detail and to see to what extent it is possible to predict reflex sensitivity if the heart rate and arterial pressure are known It confirmed our earlier finding that reflex sensitivity is more closely linked to heart rate than to arterial pressure but if it is correlated with both variables the low values of reflex sensitivity during hand grip and circulatory occlusion can be reconciled with the fact that arterial pressure is higher

The first mechanism is unlikely to be correct because prediction of the pressure and heart rate from reflex sensitivity for any type of exercise is greatly improved by specifying the type of exercise And denervation of the baroreceptors does not diminish the circulatory changes of exercise but exaggerates them (Thomas 1944 Ferrario McCubbin and Page 1969) The same argument can be applied to the third mechanism for prediction of reflex sensitivity from the circulatory parameters is improved by specifying the type of exercise and during bicycling exercise propranolol altered the heart rate and arterial pressure, but not the reflex sensitivity (*Pickering et al* 1972) Also hypocapnic hypoxia at rest changed heart rate but not reflex sensitivity (Cunningham *et al* 1972) From the present data we would therefore tentatively favour the second mechanism but in view of the fact that both exercise type and circulatory parameters appear to be important in determining reflex sensitivity it might be better in the form below



The present results indicate that the circulatory changes accompanying different types of exercise vary in their relation to one another and to the oxygen consumption The observed differences could be attributed either to the use of static and

dynamic exercise or to the use of largely proximal leg muscles for bicycling and distal arm muscles for gripping or to both (see Bevegård Freyschuss and Strandell (1966) who observed differences in the circulatory and metabolic responses to arm and leg exercise) However the statistical analysis of the relations between baroreflex sensitivity and other variables under a variety of experimental conditions allows us to conclude as follows first the relation between overall metabolic rate and baroreflex sensitivity varies with the kind of exercise used and secondly for all the states reported on in this paper not only the prevailing pulse interval but also the prevailing systolic pressure is related to baroreflex sensitivity

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The Effects of Hypoxia, Hypercapnia, and Asphyxia on the Baroreceptor-Cardiac Reflex at Rest and during Exercise in Man

By

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Abstract

Cunningham D J C E S Petersen T G Pickering and P Sleight *The effects of hypoxia hypercapnia and asphyxia on the baroreceptor cardiac reflex at rest and during exercise in man* Acta physiol scand 1972 *86* 456—465

The sensitivity of the baroreceptor cardiac reflex was tested in 12 healthy subjects by relating changes in pulse interval to pressure changes induced by phenylephrine *Hypercapnia in high oxygen* at rest was associated with a significant rise in systolic pressure and a significant fall of baroreflex sensitivity Pulse interval changed little The changes in exercise were qualitatively the same but much smaller *Hypoxia with hypocapnia* at rest was associated with a substantial tachycardia but no significant change in pressure or in reflex sensitivity In exercise the changes were similar except that baroreflex sensitivity was depressed more than by exercise itself *Asphyxia* (hypercapnia with hypoxia) at rest was associated with a combination of the changes induced by the two stimuli separately —The observed baroreflex sensitivities were compared to values predicted from the previously reported relations of reflex sensitivity to either steady state pulse interval (I_0) or the ratio of I to systolic pressure (I/I_0) Values predicted from I_0/P were in general closest to the observed values In hypoxia at rest however both I_0 and I/P_0 changed but reflex sensitivity remained unaltered showing that mechanisms exist for influencing baroreflex sensitivity and other circulatory variables independently of one another

It has recently been reported that hypercapnia especially when combined with hypoxia affects the setting of the baroreceptor—heart rate reflex system in man at any given level of systolic arterial pressure the pulse interval was shorter during the application of these respiratory stimuli (Bristow *et al* 1971 *a*) While in these studies on resting subjects the sensitivity of the reflex did not change significantly (sensitivity being defined as the lengthening of pulse interval I per unit rise of systolic pressure P ($\Delta I/\Delta P$)) we found that during bicycling the reflex sensitivity was depressed (Cunningham *et al* 1970 Bristow *et al* 1971 *b*) the degree of

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depression was related in a simple fashion to the steady state pulse interval immediately before the testing of reflex sensitivity and hence also to the work rate The general relation between baroreflex sensitivity and steady state pulse interval holds for reflex changes during change of posture at rest and in exercise (Pickering *et al* 1971) and to most but not all situations in which drugs with actions on the autonomic nerve supply to the heart are administered in rest and exercise (Pickering *et al* 1972)

In this paper we report on a study of the effects of the gaseous respiratory stimuli and bicycling separately and together on the same subjects The experiments were designed to see whether or not the relation between baroreflex sensitivity and the steady state pulse interval (or some related combination of circulatory variables) was independent of the combination of stimuli Elsewhere we report on a similar study of three kinds of exercise static and rhythmic handgrip and bicycling with the design in which the relations between exercise effort overall metabolic rate and steady state pulse interval are very different the simple relation between steady state pulse interval and baroreflex sensitivity holds for only some of these conditions (Cunningham *et al* 1972)

Methods

The quantitative approach to the study of the baroreceptor cardiac reflex which was employed in the present work has been described in detail by Smyth Sleight and Pickering (1969) and modified by Bristow *et al* (1971 *b*) Intra arterial pressure was recorded continuously through a catheter in the brachial artery Transient rises of arterial pressure (15—30 mm Hg) lasting 15—20 s were introduced by i v injections of the α sympathomimetic drug phenylephrine (50—180 μg) The reflex cardiac slowing was followed beat by beat during the pressure rise and each pulse interval I was subsequently plotted against the systolic pressure P of the preceding beat (*cf* Fig 4 and 3 of Smyth *et al* 1969) In this way a linear relation is obtained the slope of which (msec increase of I per mm Hg rise in P) is an index of baroreflex sensitivity the steeper the slope the greater the sensitivity The use of systolic rather than mean arterial pressure is discussed in the earlier papers (Smyth *et al* 1969 Bristow *et al* 1971 *b*)

Five women and six men aged 19—26 years and an older man (48) acted as experimental subjects They sat on a Krogh bicycle ergometer throughout the experiment breathing humidified gas through a low resistance valve The composition of the inspired air could be changed rapidly by a tap beyond the subject's field of vision Gas was sampled continuously at the mouth and P_{CO_2} and P_{O_2} were determined with infra red and paramagnetic analyzers (the latter modified to give breath by breath end tidal P_{O_2} according to Cunningham Kay and Young 1965) Both analyzers were calibrated frequently with chemically analyzed standard mixtures

Experimental design The states in which the determinations were made are shown in Table 1 together with an indication of the probable direction of any change from normal of the level of activity of the intracranial and the arterial chemoreceptors (*e g* Lloyd 1966) Measurements were not started until at least 5 min after a change of alveolar CO_2 (P_ACO_2) and 3 min after changes of P_{AO_2} and of exercise level

Usually 3 injections of phenylephrine were given during each state with an interval of at least 3 min between injections The coefficients of variation for these triplicate determinations were I 3 5% P 2 3% $\Delta I/\Delta P$ (slope) 11 7%

All experiments included determinations during rest and exercise in hyperoxia (a and e Table 1) in order to establish the relations between baroreflex slope ($\Delta I/\Delta P$) and I and between slope and I_0/P for these conditions Usually one or more pairs of conditions (*e g* hypocapnic hypoxia in rest and exercise b and f or hyperoxic hypercapnia c and g) were included in each experiment in an order which varied from one experiment to another in some experiments however the second member of a pair was not completed

In series e all subjects were subjected to 2—4 work loads of between 13 and 151 W In series f and g usually only one work load was employed

TABLE I Summary of experimental conditions

The figures are ranges of P_ACO_2 and P_AO_2

State	P_AO_2 torr	P_ACO_2 torr	Probable chemoreceptor activity	
			intracranial	arterial
1 *Rest*				
a hyperoxia	200–250	normal	normal	low
b hypoxia	45–55	low	low	moderately raised
c hyperoxic hypercapnia	200–250	10–12 above resting	high	low
d. asphyxia	45–55	6–10 above resting	moderately raised	high
2 *Exercise*				
e hyperoxia	200–250	normal	normal	low
f. hypoxia	45–55	low	low	moderately raised
g hyperoxic hypercapnia	200–250	10–12 above resting	high	low

Results and Discussion

Typical experimental records showing measurements made over the period of single phenylephrine induced rises of arterial pressure and plots of individual pulse intervals against the appropriate systolic pressure peaks appear in previous papers (Fig 4 and 3 of Smyth *et al* 1969 and Fig 1 and 2 of Bristow *et al* 1971 *a*)

The measurements of mean steady-state (*i e* pre injection) pulse interval I and systolic pressure P the regressions of I on P during the drug induced rises of pressure (baroreflex sensitivity $\Delta I/\Delta P$) together with the measurements of PCO PO and work load comprise the experimental data described in this paper

Fig 1 shows graphically all the determinations of I and P_o (the points) and of baroreflex sensitivity the lines arising from the points) carried out in the course of a single experiment The determinations fall into categories a b c e and g (Table I

In earlier experiments in high oxygen (Fig 3 of Bristow *et al* 1971 *b* and Fig 1 of Cunningham *et al* 1970 the progression from rest through mild and moderate exercise to heavy exercise was characterised by a progressive increase in steady-state systolic pressure P accompanied by a proportionate decrease in steady state pulse interval I on the I P diagram the progression followed an effectively linear path was downward and to the right These changes were associated with a progressive decrease in the baroreflex slope *e g* the slopes of the lines in Fig 1) Thus the harder the exercise the shorter was the pulse interval the higher the systolic pressure and the lower the reflex sensitivity In the present series attention has been directed to other states at the expense of the time which would be necessary to determine a full well-spaced set of baroreflex lines in bicycling If in Fig 1 nevertheless a straight line is imagined as joining the rest points (open circles upper left)

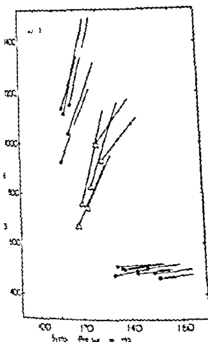


Fig 1 The whole of one representative experiment (on subject 23) shown on the pulse interval (I) systolic arterial pressure (P) diagram. Each line has the slope of the regression of I on P for one drug induced rise of pressure and is drawn to pass through the symbol to which it corresponds The symbols show the positions of the steady state (i e pre injection) values of pulse interval and systolic pressure (I and P) Open symbols denote rest closed symbols work ○ high oxygen (P_AO_2 ~ 240 torr) □ hypercapnia in high O_2 (P_ACO_2 resting +11 torr) △ hypoxia (P_AO_2 47 torr) ● work (100 and 176 watts 3 of each) in high O_2 ■ work (100 watts) in high O_2 and hypercapnia P_ACO_2 ~ 48 torr)

the exercise points (closed circles lower right) it can be seen that two out of three of the rest high-oxygen hypercapnic points (open squares) would lie above it ...cating that when exercise is used as the norm hypercapnia tends to be associated with increase of systolic pressure rather than with diminution of pulse interval The ... appears to be the case in hypocapnic hypoxia during rest (triangles) in which 2 out of 3 of the points lie below this imaginary line indicating that ...pocapnic hypoxia is associated with tachycardia rather than with change of systolic pressure In this experiment the combination of hypercapnia with exercise (black squares) produced no effects on I and P over and above those of the exercise itself

In Fig 2a the baroreflex slopes ($\Delta I/\Delta P$) for the same experiment are plotted as a function of I The line is the regression of reflex slope on steady state pulse interval for rest and exercise using only the data obtained in high oxygen without added CO_2 (open and closed circles) With this plot too the validity of drawing a linear regression line rests on our earlier work (Bristow *et al* 1971 *b*) In high oxygen work load steady state pulse interval and baroreflex sensitivity are all interrelated In certain condition however notably with static handgrip the relation of reflex sensitivity with I is not so good as another relation that includes a term for steady state systolic pressure (Cunningham *et al* 1972) Inspection of these and the present data on hypercapnia shows that reflex slope is lower when pressure is higher the combination of I and P used for the present purpose of prediction is therefore I /P

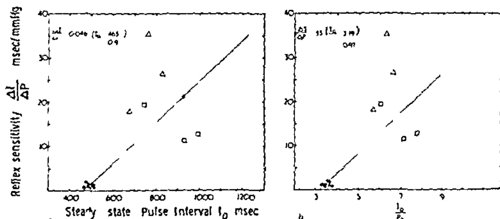


Fig 2 Data from Fig 1 replotted as baroreflex sensitivity ($\Delta I/\Delta P$) against steady state (pre-injection) pulse interval I_0 (Fig 2 *a*) and against steady state ratio I_0/P_0 (Fig 2 *b*) Symbols denote same conditions as in Fig 1 Lines are regressions of $\Delta I/\Delta P$ on I_0 (*a*) and of $\Delta I/\Delta P$ on I_0/P_0 (*b*) for determinations in rest and work during high-oxygen breathing only regression equations for these lines are shown The predictions of reflex sensitivity shown in the last two columns of Table II are means of values read off regression lines of which these two are examples Note that all the reflex sensitivities in hypoxia at rest are above the lines the reflex sensitivities in hypercapnia tend to lie below the line in *a* but are scattered around the line in *b*

In Fig 2 *b* the baroreflex slope is plotted against I_0/P_0 again the regression line is for the high-oxygen data only These 2 regression lines (Fig 2 *a b*) provide a basis for prediction of baroreflex sensitivity during conditions other than hyperoxia In the experiment illustrated in Fig 1 and 2 the effects of hypercapnia in hyperoxia were relatively greater on baroreflex slope than on pulse interval 2 out of 3 of the hypercapnia points fell below the line in Fig 2 *a* When however pressure is taken into account in the prediction (Fig 2 *b*) the two points for hypercapnia are closer to the line and the mean for the 3 lies virtually on it In hypoxia at rest on the other hand despite the considerable tachycardia there was no change of baroreflex slope in 2 of the 3 determinations and all 3 points lie well above the line in Fig 2 *a* making an allowance for pressure as well (Fig 2 *b*) makes no appreciable difference to the badness of fit

The mean values of the slopes predicted in this way for the various conditions appear beside the corresponding means of the observed slopes in Table 2 together with summaries of the other data for all 12 expts The general trends are illustrated graphically in Fig 3 and 4 The means of the predicted values of reflex sensitivity that appear in Table II are based on the separate regression lines for individual subjects In Fig 4 *a* and *b* however the regression lines have been calculated from the pooled data for all experiments There are therefore minor discrepancies between the numerical values in the table and the figures

Hyperoxic hypercapnia at rest In hypercapnia in high oxygen at rest respiratory studies indicate that the intracranial H⁺ CO_2 receptors but not the arterial chemoreceptors are activated (e g Lloyd 1966) P was increased significantly while

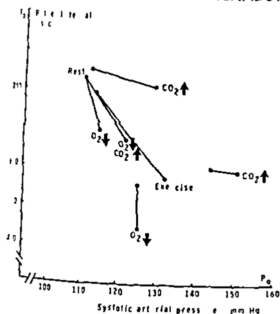


Fig 3 Mean values of steady state pulse interval I_0 plotted against mean systolic pressure P_0 for all experiments Note substantial changes with exercise in both I_0 and I At rest with hypercapnia change occurs in P rather than in Po with hypocapnic hypoxia change occurs in Io rather than in Io in asphyxia (both intracranial and arterial chemoreceptors active) there is substantial change in both I and P In exercise mean effects of hypercapnia and hypoxia separately are in the same general directions as are seen in rest

changes in I_0 were small and variable (Table II) confirming previous work from this laboratory (Bristow *et al* 1971 *a*) and the results on man of other workers (*e g* Richardson Wasserman and Patterson 1961) The significant decrease in baroreflex slope which we now find was foreshadowed in the earlier papers though in that series there was no change in mean slope Further unpublished work (M J R Lee private communication) is in agreement with the newer finding in general there is much individual variation

Hypocapnic hypoxia at rest (a situation in which the intracranial receptors are depressed by hypocapnia while the arterial chemoreceptors are only moderately active) is associated primarily with a substantial decrease of I ($p < 0\,01$) corresponding to a mean rise of heart rate from 72 to 86 beats/min On average there was only a minimal rise in P in confirmation of the work of others (*e g* Richardson *et al* 1967) We are not concerned in this paper with the mechanisms influencing the changes in heart rate and blood pressure whether primary responses from arterial chemoreceptor stimulation or secondary responses to changes in ventilation (*e g* Angell James and Daly 1969) these have been discussed by Bristow *et al* (1971 *a*) In the present experiments an unexpected new finding is the lack of a reproducible effect of hypocapnic hypoxia on baroreflex sensitivity despite the consistent tachycardia that accompanied it (Table II and Fig 4 *a* and *b*)

Asphyxia at rest (both intracranial and arterial chemoreceptors strongly stimulated) has circulatory effects which combine the predominantly hypertensive response to pure intracranial chemoreceptor stimulation with the predominantly cardio acceleratory effect of arterial chemoreceptor stimulation The baroreflex sensitivity decreased by about the amount predicted from the changes in both I and I

TABLE II Mean values of all data ± S E M Small letters in brackets after the description of the experimental conditions refer to Table I n denotes number of experiments Since there were usually 3 injections in each steady state conditions a and e for example commonly included 6 – 1? determinations of baroreflex sensitivity in each experiment The changes in I_0 P_0 and $\Delta I/\Delta P$ induced by the various experimental situations (Columns 2 3 and 4) have been tested by a t test for paired differences When significant this is indicated by x ($p < 0.05$) or xx ($p < 0.01$) The predicted reflex sensitivities shown in the two last columns are means of those calculated from the individually determined regressions (cf Fig 2a and b) Where predicted values were significantly different (Wilcoxon s test) from observed this has been indicated by x ($p < 0.05$) or xx ($p < 0.01$)

Condition	n	I_0 msec	P_0 mm Hg	Reflex sensitivity $\Delta I/\Delta P$	Reflex sensitivity predicted from	
					I_0	I_0 P_0
Rest hyperoxia (a)	12	778 ±46	114 ±4	17 6 ±2 5		
Exercise hyperoxia (e)	12	563xx ±31	132xx ±5	6 8xx ±1 2	6 8	6 [illegible]
Rest hyperoxia (a)	8	8?8 ±73	112 ±3	16 0 ±2 9		
Rest hypocapnic hypoxia (b)	8	[illegible]01xx ±59	116 ±5	16 2 ±2 5	11 3xx	10 7xx
Rest hyperoxia (a)	7	853 ±71	114 ±8	19 ? ±3 9		
Rest hyperoxic hypercapnia (c)	7	812 ±60	130xx ±8	14 5x +2 3	17 1x	13 5
Rest hyperoxia (a)	6	82? 65	112 ±10	20 1 4 3		
Rest asphyxia (d)	6	675xx ±64	123x ±8	12 6x ±2 0	1? C	11 4
Exercise hyperoxia (e)	7	55 ±34	12[illegible] 5	7 1 ±1 3		
Exercise hypocapnic hypoxia (f)	7	451xx 25	1 6 [illegible]	4 3xx ±1	3 5	4 1
Exercise hyperoxia (e)	4	605 6?	145 1	6 [illegible]		
Exercise hyperoxia hypercapnia (g)	4	594 58	151 11	5 2 ±? 5	7 ?	6 3

these changes were all significant and are like those previously reported (Table II of Bristow *et al* 1971 *a*) They are also like the response to exercise (Fig 3 and 4)

In all the three conditions just described the baroreflex is obviously re set in the sense that the steady state values of I_o and P_o when plotted on the I P diagram lie well away from the baroreflex line at rest or its extension (*e g* Fig 1 see also Fig 3) Bristow *et al* (1971 *a*) were able to combine the changes in I_o and P_o in a single quantity I that is the pulse interval at a single reference arterial pressure and thus to express the effects of hypercapnia on the reflex more quantitatively The validity of their treatment depended on the absence of major change of baroreflex slope their conclusions were thus virtually independent of the value chosen for the reference systolic pressure In the present series the dramatic changes of slope with exercise rule out this simple approach

Exercise and gaseous stimuli The results of the combination of the gaseous stimuli with exercise also appear on the I P diagram in Fig 3 CO inhalation in high oxygen which is unlikely to increase the activity of arterial chemoreceptors whilst adding to intracranial stimulation had qualitatively the same primarily pressor effect as during rest but the magnitude was strikingly less and the changes were not significant perhaps because of the great peripheral vasodilation already existing in exercise or perhaps because of some change in the central effect Hypoxia with hypocapnia was without effect on arterial pressure but reduced pulse interval substantially While intracranial chemoreceptor activity was probably minimal the exact effect of hypocapnic hypoxia on arterial chemoreceptors in exercise is uncertain Hypoxia is undoubtedly a much stronger stimulus to breathing in exercise than at rest (for references see Cunningham Spurr and Lloyd 1968) but the potentiation of the hypoxic drive could occur in the respiratory centre and not involve any part of the circulatory control system

Both central and arterial chemoreceptor activation in exercise was associated with a reduction of baroreflex sensitivity The margin available for changes was small in both states but the reduction of slope was nevertheless significant in the case of hypoxia in exercise, this contrasts with the lack of change of slope associated with hypoxia at rest why arterial chemoreceptor activation by itself should be without effect on sensitivity in the absence of exercise is not clear

To the best of our knowledge there have been no studies in exercise comparable with those reported here though Asmussen and Nielsen (1955) reported on the effects of hypoxia combined with exercise on some other circulatory variables

The relation of baroreflex sensitivity to I_o and P_o

In Fig 4 *a* and *b* we have represented the correlation between changes in reflex sensitivity and other circulatory variables Previously (Bristow *et al* 1971 *b*) we found a good correlation between changes in sensitivity (slope) and the degree of tachycardia (I) induced by exercise in hyperoxia It is clear however particularly in static exercise (Cunningham *et al* 1972) that other circulatory parameters ha to be considered too Static exerci e and bicycling considered together and n

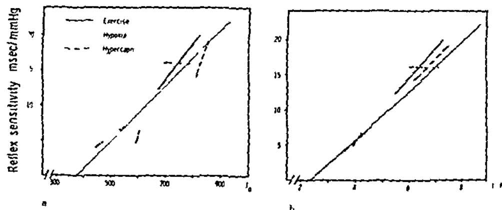


Fig 4 *a* and *b* Mean changes in baroreflex sensitivity shown as arrows on (*a*) $\Delta I/\Delta P$ versus I_0 and (*b*) $\Delta I/\Delta P$ *vs* I_0/P_0 diagrams The continuous diagonal lines are the regressions for rest and exercise in high-oxygen breathing only (pooled data from all experiments) ——→ Hypercapnia in high O_2 → Hypoxia with hypocapnia ——→ Asphyxia. All arrows conform more closely to the regression line in *b* than in *a* with the notable exception of that denoting hypocapnic hypoxia at rest (only arterial chemoreceptors active) which conforms to neither

combination of hypercapnia and bicycling are fitted better when baroreflex slope is expressed as a function of both systolic pressure and pulse interval (I_0/P_0) Until now it has been uncertain whether the circulatory changes in I_0 and P_0 might be linked in a constant way with the setting and sensitivity of the baroreflex control of heart rate It now appears that the linkage is not always quantitatively the same since at rest arterial chemoreceptor stimulation in isolation changes both I_0 and I_0/P_0 equally without any change in reflex sensitivity (Fig 4 *a* and *b*) Similarly Fig 10 in the review by Korner (1971) indicates that in unanesthetized rabbits the baroreflex sensitivity in hypocapnic hypoxia is unrelated to concurrent tachycardia or bradycardia Korner has no corresponding exercise data on these rabbits with which to contrast the absence of slope change in hypoxia In our subjects the strong arterial chemoreceptor activity occurring in asphyxia at rest which is accompanied by increased rather than decreased intracranial (H CO) receptor activity is associated with the predicted change in baroreflex sensitivity (Table II) this may be due merely to the opposing tendencies of central hypercapnia and of arterial chemoreceptor stimulation to produce respectively more than and less than the expected depression of baroreflex sensitivity (Fig 4 *a*)

These findings in general might indicate that exercise is associated with inhibition of the baroreceptor-cardiac reflex which is not seen in hypoxia an inhibition perhaps specific to exercise Alternatively if the inhibition of the reflex is non specific related perhaps to changes in vagal and/or sympathetic tone at the sinus node arterial chemoreceptor stimulation might be associated with facilitation of the reflex In fact Euler and Liljestrand (1943) showed that the effect of carotid occlusion on blood pressure in cats was enhanced during hypoxia

The situation is complex but it is clear that reflex setting can be changed independently of reflex sensitivity

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Pulmonary Hypertension during Systemic Hypotension Effects of Oxygen Breathing and of Vasodilators

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Abstract

Bo G A Halge and J Hognestad *Pulmonary hypertension during systemic hypotension Effects of oxygen breathing and of vasodilators* Acta physiol scand 1972 86 466—473

Hemorrhagic hypotension in cat has been found to cause pulmonary arterial hypertension The purpose of the present work has been to investigate whether pulmonary vasoconstriction is involved in this response In the third hour of maintained standardized systemic hypotension the vasodilating agents bradykinin and isoproterenol were infused into vena cava inferior or a period of ventilation with 100 % oxygen was inserted Pulmonary arterial and left atrial pressures as well as cardiac output were recorded Both the vasodilating drugs and the ventilation with oxygen were able to reduce the pulmonary vascular resistance response by one third We conclude that the pulmonary hypertension developed during systemic hypotension in cat is partly caused by active vasoconstriction Such a vasoconstriction could well have been elicited by regional pulmonary hypoxia due to closure of terminal airways The main contributing factor to this particular type of pulmonary hypertension is apparently a mechanical obstruction of lung vessels

In cats exposed to hemorrhagic standardized systemic hypotension the pulmonary arterial pressure has been found to increase gradually throughout a 3 h period to about 70 per cent above the initial value in spite of a 50 per cent reduction in cardiac output (Bo and Hognestad 1971) Animals made thrombocytopenic did not demonstrate such an increase in pulmonary arterial pressure (Bo and Hognestad 1971 a finding which suggests platelet aggregation and embolism to be involved in the response

Pulmonary microembolism by platelet aggregates (Bo and Hognestad 1972) as well as by other materials (Halmagyi and Colebatch 1961) has been found to cause pulmonary arterial hypertension Mechanical plugging of small pulmonary arteries as well as reflex-elicited pulmonary vasoconstriction are believed to contribute to this response (Halmagyi and Colebatch 1961 Dexter 1965) The response pattern appears to depend largely on the size and the number of the emboli

The purpose of the present work has been to investigate whether pulmonary vasoconstriction is involved in the pulmonary arterial hypertension which develops

subsequent to a hemorrhage. Attempts to dilate the pulmonary vasculature were made by using vasodilating agents and by ventilating with oxygen at ambient pressure.

Methods

Anesthesia and experimental procedure. We anesthetized cats (2.9 to 3.6 kg) with sodium pentobarbitone (30 to 35 mg/kg), made a tracheostomy, induced muscular paralysis with Alloferin® (0.5 mg/kg) and ventilated the animals by intermittent positive pressure. A ventilation frequency of 21/min was chosen, and the tidal volume was adjusted so as to maintain arterial pH between 7.38 and 7.42 until the start of the bleeding, whereafter tidal volume was kept constant. The animals were given heparin, 500 I.U./kg, intravenously. By keeping the animals on a heated table and enclosed within a transparent polyethylene tent with warm humidified air entering it, we were able to maintain deep rectal and lung surface temperatures constant and in the normal range.

The thorax was opened by a sternum-splitting incision, and polyethylene catheters were introduced into the femoral artery, the pulmonary artery and the left atrium for recordings of the femoral arterial pressure (P_{FA}) with a Statham P23Gb transducer, the pulmonary arterial pressure (P_{PA}) with a Statham P23Db transducer and the left atrial pressure (P_{LA}) with a Statham P23De transducer. Cardiac output was measured by the use of a wraparound flow transducer on the ascending aorta, connected to a Nycotron square wave electromagnetic flowmeter (type 372, Nycotron A/S, Norway). The pressures and the flow were recorded on a polygraph (Model 350, Sanborn Co., California). The mean pulmonary vascular resistance (PVR) was calculated by dividing the pressure difference $P_{PA}-P_{LA}$ with the cardiac output.

pH measurements in arterial blood were carried out with a Radiometer pH meter 27.

Arterial blood oxygen tension was measured with the use of a Po_2 electrode (type E 5046) connected to a Radiometer Acid Base analyzer (type PHM 71) equipped with a Po_2 module.

Intravenous infusions were carried out through a polyethylene catheter which was pushed through the femoral vein in the cephalad direction until the catheter tip was situated in the inferior vena cava. For infusions we used a Harvard infusion pump (model 947).

Standardized hemorrhagic hypotension was induced by bleeding the animal through the femoral artery into a graded syringe until mean femoral arterial pressure had decreased to a level between 50 and 60 mm Hg. Femoral arterial pressure was maintained at this level by appropriate small extra withdrawals or infusions of blood.

The drugs used were taken from the following stock solutions:

Nembutal® Abbott 60 mg/ml
Alloferin® Roche 5 mg/ml
Heparin® AL Oslo 1 mg/ml
Isoprenalin Rikshospitalets apotek Oslo 0.2 mg/ml
Acetylcholine® Roche 100 mg/amp
Synthetic Bradykinin Sandoz 0.1 mg/ml

Results

Control animals. Intravenous infusions of bradykinin were carried out for 1 min periods in 3 non-bled and normotensive animals in doses varying from 4 to 10 μg/kg/min. The mean pulmonary arterial pressure response of 12 infusions was +1.5 mm Hg, the range being from 0 to 3 mm Hg. The hemodynamic effects of two such infusion experiments are demonstrated in Fig. 1.

In one animal bradykinin (8 μg/kg/min) was infused for 1 min before and for 1 min after occlusion of the left pulmonary artery. The occlusion caused a 4 mm Hg elevation of pulmonary arterial pressure, but no change in left atrial and systemic arterial pressures. Cardiac output also remained constant. The effects of the superimposed bradykinin infusion was a further rise of 3 mm Hg in pulmonary arterial pressure, a 30 per cent rise in cardiac output, a 30 mm Hg reduction in syst

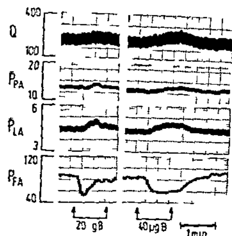


Fig 1 Effects in a non bled control animal of two 1 min i v infusions of 20 and 40 µg bradykinin. Q mean cardiac output (ml/min) P_{PA} mean pulmonary arterial pressure P_{LA} mean left atrial pressure P_{FA} mean femoral arterial pressure The pressures are given as mmHg

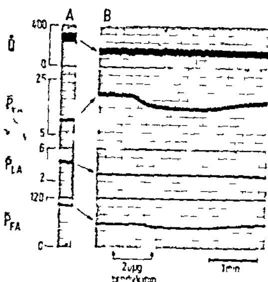


Fig 2 Effects of an infusion of bradykinin in a cat with pulmonary hypertension developed subsequent to a blood lost Panel A shows the values for flow and pressures before the bleeding Panel B shows the values in the third hour of maintained hemorrhagic hypotension Symbols and abbreviations as in Fig 1

arterial pressure and a 0 5 mm Hg rise in left atrial pressure The responses were thus similar to those shown in Fig 1

Acetylcholine in doses from 0 3 to 2 µg/kg infused for 1 min gave varying results The smallest dose gave a slight fall in pulmonary arterial pressure without any change in cardiac output The larger doses reduced the cardiac output and did as a rule cause a transient rise in the calculated pulmonary vascular resistance value

Bled animals In a group of 11 animals the standardized hemorrhagic hypotension (P_{FA} = 50 to 60 mm Hg) was maintained for 3 h The immediate effect of bleeding was a fall in pulmonary arterial and left atrial pressure as well as a fall in cardiac output During the second and third hour however the pulmonary arterial pressure increased above initial level whereas cardiac output remained low The development here was as has been reported earlier (Bo and Hognestad 1971)

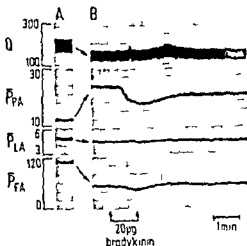


Fig 3 Effects of an infusion of bradykinin in a cat with pulmonary hypertension developed subsequent to a blood loss Panel A shows the values for flow and pressures before the bleeding Panel B shows the response in the third hour of maintained hemorrhagic hypotension Symbols and abbreviations as in Fig 1

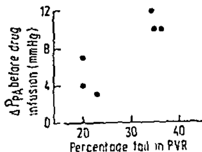


Fig 4 The maximal PVR responses to bradykinin in each of 6 bled animals with pulmonary hypertension The responses are plotted against the degree of P_{PA} rise seen at time of tests 3 h after establishing hemorrhagic hypotension The excess PVR above pre bleeding value is set to 100 per cent

In 6 animals intravenous infusions of bradykinin (3 to 19 µg/kg/min) were carried out during the period with pulmonary arterial hypertension In each animal the dose was increased until a maximal response was achieved Examples are shown in Fig 2 and 3 In these tests the infusions of 20 µg bradykinin caused a marked reduction of the increased pulmonary arterial pressure This effect occurred in spite of none or a small rise only in cardiac output

The animals which developed the highest pulmonary arterial pressure rise during the hemorrhagic period also responded with the largest pressure reduction to bradykinin This is shown in Fig 4 where pulmonary arterial pressure rise before infusion of bradykinin is plotted against percentage fall in PVR caused by the infusion

Bradykinin infusions did not alter the cardiac output in the animals where the relative reduction of this parameter had been 50 per cent or more by the bleeding (Fig 2) When, however cardiac output had been only moderately reduced by the bleeding the infusions of bradykinin caused some rise in aortic flow (Fig 3) A similar response pattern could also be demonstrated for the mean systemic arterial pressure At a femoral arterial pressure of 50 to 60 mm Hg very little further reduction was caused by infusions of bradykinin After retransfusion larger absolute

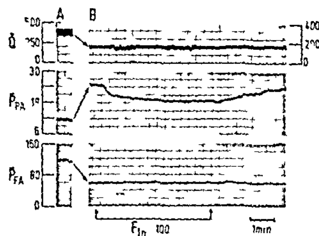


Fig 5 The effect of oxygen breathing on the pulmonary hypertension which develops during a period of systemic hypotension Panel A shows the values for flow and pressures before the bleeding Panel B shows the values in the third hour of maintained hemorrhagic hypotension Symbols and abbreviations as in Fig 1

and relative responses to bradykinin were seen Even in this situation the fall in systemic arterial pressure was however smaller than in non bled animals with comparable levels of cardiac output and femoral arterial pressure

Infusions of acetylcholine for 1 min in doses from 0 3 to 1 5 µg/kg resulted in a fall in cardiac output and in pulmonary arterial pressure We were unable to find a dose and an infusion rate which would give responses from the pulmonary vascular only

In 9 bled animals the ventilation gas was temporarily changed from room air to 100 per cent oxygen An experiment of this type is demonstrated in Fig 5 Here oxygen breathing resulted in a reversible reduction by 6 mm Hg in mean pulmonary arterial pressure and in a calculated fall in excess PVR of 24 per cent Systemic arterial pressure and cardiac output did not change Isoproterenol (? µg/kg/min) infused for 5 min 15 min after the end of oxygen breathing gave the same calculated PVR reduction

Fig 6 gives the pulmonary arterial pressure responses to all tests with oxygen breathing in bled animals The responses are plotted against the arterial oxygen tension (Pa_{O_2}) determined just before the change of ventilation gas from room air to 100 per cent oxygen The diagram demonstrates that none of the animals were gravely hypoxic and that there was a tendency towards smaller responses to oxygen breathing as the pre test Pa_{O_2} values approached the normal value

Since this pulmonary vasodilator effect of oxygen breathing in animals with near normal Pa_{O_2} was somewhat surprising we examined P_{O_2} also in mixed venous blood drawn from the pulmonary artery We found $P\bar{v}_{O_2}$ to be mean 32 mm Hg (n 5)

Oxygen breathing in 3 non bled animals did not elicit any detectable changes in the hemodynamic parameters recorded

Discussion

Intravenous injection of vasodilating agents has previously been used to analyze pulmonary vascular reactivity in states of pulmonary hypertension and also after

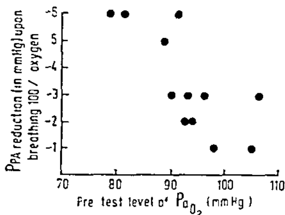


Fig 6 Decreases in pulmonary arterial pressure upon breathing 100 per cent oxygen in 5 bled cats which developed pulmonary arterial hypertension (see text) Each oxygen induced decrease in P_{PA} is plotted against the value for arterial oxygen tension (P_{aO_2}) found in that particular animal just before the test was carried out

experimental pulmonary embolization It has thus been shown that the pulmonary vascular pres or responses to microembolization with barium sulphate can be abolished by simultaneous infusions of isoproterenol (Halmagyi and Colebatch 1961) Similarly the pressor response to alveolar hypoxia can be reduced or abolished by infusions of bradykinin (Segel *et al* 1970) or of acetylcholine (Harris *et al* 1956) Results of this type may give information as to the extent to which an increased vascular smooth muscle tone is involved in the pulmonary hypertension However such use of vasodilator drugs can hardly be expected to disclose the intrinsic nature of a pulmonary vasoconstriction

A critical point to be taken into consideration is the indirect passive effects upon the pulmonary vascular bed of changes in cardiac output or in the systemic circulation Ideally a vasoactive drug which is inactivated before it reaches the left atrium should be employed (Borst *et al* 1956) We believe that the small increase in pulmonary arterial pressure regularly seen after bradykinin infusions in our control group of animals was a consequence of the transient rise in cardiac output caused by the drug Bradykinin has been reported to cause vasoconstriction in isolated perfused lung preparations (Hauge Lunde and Waaler 1966) However this is not the typical response in normal lungs *in situ* (Segel *et al* 1970)

In all our bled animals the pulmonary arterial pressure increased above the initial level in the second and third h of hemorrhagic systemic hypotension in spite of the concomitant fall in cardiac output In animals in which the pulmonary arterial hypertension after hemorrhage was small or moderate bradykinin caused either no effect on pulmonary arterial pressure or small reductions only This pressure reducing effect was more pronounced however in animals with larger post bleeding increases in pulmonary arterial pressure

In bled animals bradykinin did either not affect cardiac output or it caused a moderate increase in this parameter Since left atrial pressure remained unchanged the fall in pulmonary arterial pressure caused by the infusions must therefore have been due to pulmonary vasodilatation and fall in PVR The excess PVR which built up during the hypotensive period could at most be reduced by 35 per

when bradykinin was infused No further effect was obtained by increasing the dose One can therefore conclude that pulmonary vasoconstriction did at least contribute with one third of the PVR increase in this particular type of pulmonary hypertension Mechanical plugging by pulmonary thromboemboli may also be involved A third factor could be regional vascular collapse caused by the transient very low pulmonary arterial pressure which occurs during the first h of systemic hypotension

Bradykinin has been found to be a potent vasodilating agent in the systemic circulation, an observation which could readily be demonstrated in the non bled control animals In the hypotensive animals however, this response was abolished or almost abolished The lost peripheral response may partly be due to a general decrease in precapillary systemic vascular tone which has been found to develop after hemorrhage and which is presumably caused by accumulation of local vasoactive metabolites (Mellander and Lewis 1963) Another factor may be the low vascular distending pressure in the bled animals In 1902 Bayliss pointed out that the intravascular pressure participates in determining the degree of vascular smooth muscle contraction through the distension it causes in the vessel wall The relationship between transvascular pressure and vessel responsiveness to various stimuli was later outlined by Folkow (1953)

On the other hand the Bayliss phenomenon does not seem to play an important role in the pulmonary circulation Almost identical pressor effects of bradykinin were seen before and after occlusion of one pulmonary artery with a mechanical increase in the pulmonary arterial pressure

Alveolar hypoxia is a vasoconstrictor stimulus in the pulmonary circulation in the cat but the fraction of oxygen in the inspired gas has to be lowered to 12 per cent or less in order to obtain such a response (Hauge and Staub 1969) This threshold represents an arterial oxygen tension of about 55 mm Hg With this information in mind it was somewhat surprising to find that ventilation with 100 per cent oxygen caused a considerable reduction in the pulmonary arterial pressure in the bled animals since the arterial oxygen tension in all the cases was much higher than 55 mm Hg prior to the oxygen test One explanation for the effect of oxygen breathing could be a very low perfusion through hypoxic areas of the lung which would give a high arterial oxygen tension in spite of regional pulmonary hypoxia

Some regional alveolar hypoxia may have been caused by pulmonary platelet microembolism Experimental pulmonary microembolism has been shown to cause peripheral airway constriction and systemic arterial hypoxia (Nadel *et al* 1964) In the present preparation a fall in dynamic compliance is regularly seen during the second and third h after the hemorrhage an observation which we have interpreted mainly as being due to closure of terminal airways (Bo and Hauge 1972)

Also a very low pulmonary arterial P_{O_2} may play a role In an isolated cat lung preparation Bergofsky *et al* (1968) found that pulmonary vasoconstriction could be elicited by lowering the pulmonary arterial P_{O_2} to 25 mm Hg even when the alveolar P_{O_2} was maintained above 100 mm Hg We found the pulmonary arterial P_{O_2} in bled animals to be as low as 22 mm Hg It has previously been demonstrated

that pulmonary arterial hypoxia acts synergistically with a moderate alveolar hypoxia in causing pulmonary vasoconstriction (Hauge 1969) Mechanisms of the above type go a long way to explain the observed effect of oxygen breathing in our bled cats with their near normal Pa_{O_2} values Furthermore the pulmonary vasoconstrictor response to hypoxia is potentiated by a fall in blood pH Such a potentiation presumably occurred in the present preparation since here arterial pH in the bled animals fell from normal value to a mean of 7 08 (range 7 14—6 98) in the course of the 3 h hypotensive period

The effect of oxygen breathing on the increased pulmonary arterial pressure of the bled cats was of the same magnitude as the effects of vasodilator agents

This finding indicates that local pulmonary hypoxia could be a dominant factor in the increased pulmonary vascular tone seen in hemorrhagic hypotension

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Response to Submaximal and Maximal Exercise at Different Levels of Carboxyhemoglobin

By

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Abstract

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Five subjects performed submaximal and maximal bicycle and maximal treadmill exercise in normalcy and after carbon monoxide inhalation giving different levels of carboxyhemoglobin (COHb) in the blood During maximal treadmill exercise work time on a fixed work load and maximal oxygen uptake were decreased with increasing level of COHb ($r = 0.79$ and $r = 0.85$ respectively) Peak blood lactate concentration and pulmonary ventilation were unchanged Highest measured heart rate was lower in parallell with the increased COHb level compared to control studies During submaximal work heart rate was increased and oxygen uptake was unchanged at the various levels of COHb At low submaximal work loads blood lactate concentrations and oxygen deficit was unchanged but increased as work load and COHb-level increased

Key words Submaximal and maximal work carbon monoxide oxygen uptake heart rate lactate concentration pulmonary ventilation physical performance

The physical performance capacity and maximal oxygen uptake (V_{O_2}) can be affected by altering factors in the oxygen transport system When arterial oxygen content is reduced as during acute hypoxia the amount of oxygen offered to the tissues during maximal exercise is reduced The maximal cardiac output is not changed compared to normoxia and therefore max V_{O_2} is decreased in parallell with the decreased hemoglobin oxygen saturation (Sternberg *et al* 1966) Partial blocking of the hemoglobin (Hb) by carbon monoxide (CO) can induce a similar reduction in the arterial oxygen content but without affecting the arterial oxygen tension The aim of the present study was to explore some of the effects of carboxyhemoglobin (COHb) on the cardio respiratory system during submaximal and maximal exercise Of special interest was the maximal work level It is a well known fact that the capacity for physical work requiring large muscle groups for longer than 2 to 3 min depends mainly on aerobic metabolism If oxygen transport capacity of the blood is one of the main limiting factors in such work situations reduction of this capacity should be correlated with reduction of physical work performance and max V_{O_2}

[1] Roger Huot is on leave from L Institute de Cardiologie de Montreal supported by a Fellowship from the *Samuel McLaughlin Foundation*

While completing our study two communications (Pirnay et al 1971 and Vogel et al 1971) involving maximal exercise during CO exposure were published. Only one level of COHb was mentioned.

Subjects, Methods and Procedures

Subjects

Ten well trained healthy physical education students or teachers age between 22 and 34 years (mean age 27.5 years) and with a maximal oxygen uptake of between 3.80 liters/min and 4.97 liters/min (mean 4.36 liters/min) volunteered for this study. Five of these subjects performed both submaximal and maximal loads, the other 5 maximal work loads only.

Methods

Submaximal exercise tests were performed on a mechanically braked Monark bicycle ergometer. Maximal exercise was done both on a motor driven treadmill and on the bicycle ergometer. Heart rate (HR) was obtained from the electrocardiogram recorded for 15 s each min during submaximal work loads and continuously near the end of maximal exercise. The average value for HR during the fifth and sixth min of submaximal exercise and the peak value during maximal exercise are given in the Results. Submaximal tests were of 6 min duration. All tests were of constant intensity and the same load was used in control and as well as CO experiments. The load was choosen as to exhaust the subjects on control days in about 5-6 min. Levelling off of the oxygen uptake ($\dot{V}_{O_2}$) with increasing work load was the criterium for maximal oxygen uptake (max $\dot{V}_{O_2}$). Expired air was collected in Douglas bags. At least 2 bags were obtained during the last 2 min of each work load. Volume of expired air was measured in a balanced Tissot spirometer. Gas samples were analysed with a modified Haldane technique. Samples for blood lactate concentration were obtained from a prewarmed fingertip and analysed according to the method of Baker Summerson modified by Strom (1949). Total hemoglobin (THb) was determined with the alveolar CO method as described by Sjöstrand (1948) later slightly modified by Cederlund et al (1966). The CO for the experimental exposure was withdrawn from a bomb and introduced with a syringe into a close circuit breathing apparatus to which the subject was connected and into which oxygen was fed as it was utilized. The subject rebreathed the CO mixture for 15 min. After 5 min of breathing room air blood samples for determination of the blood COHb concentration were obtained from an antecubital vein through an indwelling catheter and analysed on a CO-oximeter (Instrumentation Laboratory Inc. Model 182). The blood samples for COHb were drawn at rest and after the first CO breathing immediately before the first submaximal work load, immediately before and after the second submaximal work load and again immediately before and after the maximal bicycle test. Blood samples for determination of the COHb level during the treadmill test were taken immediately before and after the run. The COHb level for a given exercise test was taken as the average of the values immediately before and after the test. Oxygen deficit (O_2 def) was measured as describd by Karlsson (1971) and calculated as the difference between the theoretical total oxygen requirements with complete aerobic energy yield and measured oxygen uptake during the whole work period. The significance of the difference between control and "experimental" results was evaluated by the t test for correlated samples. Correlation coefficients were calculated by Pearson's product moment method. The following statistical symbols were used: $\bar{X}$ = Mean, SD = standard deviation, r = Correlation coefficient, $\bar{D}$ = difference between 2 means. $\bar{D}$ was accepted as significant if ≤ 0.05 and it was accepted as probably significant when $p \leq 0.1$ if 1 or 2 degrees of freedom more would have lowered the p to $t \leq 0.05$.

Comments

The levels of COHb selected for the exposure in the first part of the study were 7 % as a low exposure level (7 % COHb) and 20 % as a moderate exposure level (20 % COHb). Additional levels of COHb below 7 % and intermediate between 7 % and 20 % were also obtained. The theoretical amount of CO necessary to attain these levels were established as follows. Assuming that the total CO transporting capacity of the blood was the product THb × 1.34 (ml) then the amount of CO necessary to bind with respective 7 % and 20 % of the THb was obtained as the product THb × 1.34 × 7/100 and THb × 1.34 × 20/100 (ml).

Preliminary experiments indicated that this theoretical amounts had to be increased by about 25 %. We attributed this fact partly to myoglobin and partly to the limited rebreathing period of 15 min not allowing full equilibrium to be reached between blood and administered CO. During these preliminary experiments we also established the degree of COHb decrease that occurred during the two submaximal work loads. The compensatory amount of CO adequate to bring the COHb back to its preexercise levels before the maximal test was determined using the formula Compensatory amount = (THb × 1 34) (Pre submaximal exercise COHb-Post submaximal exercise % COHb). Again this theoretical amount had to be elevated slightly.

Procedure

This study consists of 2 parts. In the first part 5 of the 10 subjects carried out submaximal bicycle and maximal bicycle and treadmill work in normalcy and at 2 levels of COHb (7 % and 20 % respectively). In part 2 all the 10 subjects performed only maximal treadmill exercises in normalcy and at 3 different levels of COHb (varying between 4 8 % and 21 2 %).

In each of this 5 subjects in the first part of this study baseline values were obtained for max $\dot{V}_{O_2}$ at work loads representing 30 % and 70 % respectively of max $\dot{V}_{O_2}$. These work loads will subsequently be referred to as low submaximal exercise (30 % WL) and high submaximal exercise (70 % WL). At the rate of 1 exp every fourth or fifth day the following schedule was followed at a COHb level of 7 % on one day a 30 % WL, a 70 % and a bicycle maximal work load test (max WL) were performed while on a later day one treadmill max WL was performed. The same routine was followed at a COHb level of 20 %.

On bicycle days the procedure was as follows (1) a 15 min period during which the supine subjects were loaded with CO (2) 5 min of rest to equilibrate alveolar air with room air (3) the low submaximal work load on the bicycle ergometer was performed for 6 min (4) 5 min rest period sitting on the bicycle ergometer (5) the high submaximal work load was performed for 6 min (6) 15 min of supine rest breathing compensatory dose of CO (7) the maximal bicycle work was performed.

On treadmill days the procedure was as follows (1) a warming up period of 15 min during which they ran at half the speed of the maximal run (2) a 15 min period during which the CO was administrated (3) the maximal run was then performed.

) One to three blood samples for lactate determinations were taken after the end of each submaximal and maximal work test.

In the experiments during the control days which in all cases were performed before the CO experiments the procedure was exactly the same as during the days with CO exposure except that the CO administration periods were replaced on control days by resting periods of equal duration. On control days no blood for COHb determination was drawn. Thus as much as possible the experimental protocol for control and CO days was standardized so as to exclude any variation factor except CO administration.

Results

Mean values from experiments in the first part of this study are presented in Table I.

Low submaximal exercise (30 % WL)

The attained mean COHb levels at each exposure level were 7 1 % and 20 1 % respectively. Compared to control data $\dot{V}_{O_2}$ was unchanged at both exposure levels and mean oxygen deficit (O def) and lactates (HLa) showed only minor variations. $\dot{V}_E$ was increased at both exposure levels but not significantly; the mean HR showed a small (5 beats/min) unsignificant increase at 7 % COHb and a larger (14 beats/min) significant ($p < 0.02$) increase at 20 % COHb compared to control data.

High submaximal exercise (70 % WL)

The attained mean COHb levels at each exposure level were 6 2 % and 17.2 % respectively. Compared to control experiments mean $\dot{V}_{O_2}$ was unchanged; mean

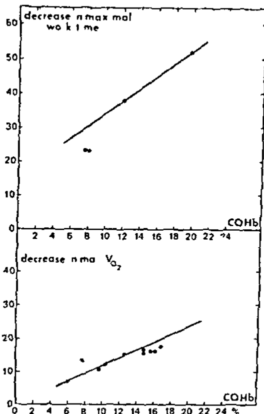


Fig 1 Relation between different levels of COHb in blood and corresponding per cent decrease in work time on a fixed maximal rate of work on the treadmill (top) and per cent decrease in maximal oxygen uptake (lower part) The equations for the regression lines are y = 15 8 + 1 87 and y = −0 19 + 1 17 × respective ly

g and HR both increased significantly at both exposure levels Mean O def and were essentially unchanged at 7 % COHb compared to control experiments Th O def increased from 1 7 l during control days to 2 1 l at 20 % COHb the increase being probably significant ($p < 0.1$) Mean HLa increased markedly at 20 % COHb compared to control data ($p < 0.001$)

Maximal exercise (max H L)

) *Bicycle experiments* The attained mean COHb concentration at each exposure els were 7 5 % and 20 7 % respectively Mean work time decreased from 4 min 11 s during control experiments to 3 min 14 s at 7 % COHb At 20 % COHb the mean work time was reduced to 2 min 27 s These changes were highly significant ($p < 0.001$) Mean max V_{O_2} decreased from 3 64 liters/min on control days to 3 30 liters/min (difference 9 3 per cent) at 7 % COHb ($p < 0.001$) and to 2 85 liters/ (difference 21 8 per cent) at 20 % COHb ($p < 0.001$) Compared to experi on control days mean V_E remained essentially unchanged at both exposure levels and mean HR decreased by 2 beats/min at 7 % COHb and by 4 beats/min at the 20 % COHb Though small this latter decrease was significant ($p < 0.02$)

TABLE I Cardiorespiratory and metabolic measurements in five subjects during submaximal and maximal exercise on the bicycle ergometer and maximal work on the treadmill in n c males at two levels of CHOb

Bicycle												
	low submaximal load						high submaximal load					
Subjects Control	CoHb sat	O D I	$\dot V_{O}$ L/min	HR Beats/min	$\dot V_E$ L/min	HLa mM	COHb sat	O D I	$\dot V_{O}$ L/min	HR Beats/min	$\dot V_E$ L min	HLa mM
I S	NM	0 34	1 14	89	27 5	1 7	NM	1 49	2 37	143	61 7	4 6
B S	NM	0 26	1 22	92	29 0	2 1	NM	1 34	2 73	140	66 5	7 4
I H	NM	0 74	1 26	80	24 6	2 5	NM	2 20	2 96	141	68 0	6 9
U B	NM	0 08	1 09	96	26 9	2 5	NM	1 50	2 56	164	59 7	4 3
B L	NM	0 41	1 11	113	24 6	1 8	NM	2 02	2 47	163	56 1	6 3
Mean		0 37	1 16	94	26 9	2 1		1 71	2 61	150	62 7	5 0
S D		0 24	0 07	12 14	2 2	0 5		0 38	0 25	12	4 9	2 5
7 COHb												
I S	6 9	0 64	1 22	103	32 5	1 5	6 5	1 32	2 42	151	67 6	5 1
B S	7 8	0 36	1 18	91	28 7	2 0	6 6	1 75	2 76	142	72 5	4 4
I H	7 2	0 16	1 18	84	30 5	1 5	6 3	1 76	2 90	145	70 8	5 5
U B	6 8	0 16	1 16	105	23 9	2 9	5 6	1 32	2 56	166	62 1	3 4
B L	6 9	0 20	1 02	112	24 8	1 2	5 9	2 01	2 41	168	58 4	7 2
Mean	7 1	0 30	1 15	99	28 5	1 9	6 2	1 63	2 61	154	66 3	5 1
S D		0 21	0 08	11 29	3 2	6 4	–	0 30	0 22	12	5 92	2 0
20 COHb												
I S	19 6	0 34	1 15	107	35 9	3 0	15 6	1 70	2 31	158	63 3	7 0
B S	20	0 40	1 13	96	31 7	1 2	17 2	1 74	2 66	153	78 8	5 5
I H	21	0 37	1 26	98	38 3	3 4	18 7	2 62	3 07	164	81 1	8 8
U B	20	0 53	1 20	118	25 2	1 2	17 2	2 03	2 58	177	75 3	6 5
B L	19 8	0 44	1 08	123	27 5	2 6	17 1	1 92	2 34	176	64 0	7 7
Mean	20	0 41	1 16	108	31 7	2 3	17 2	2 00	2 59	166	75 1	7 1
S D		0 08	0 07	11 99	5 5	1 0	–	0 37	0 31	11	7 44	1 8

* Expressed by speed in km per hour slope in degree
NM not measured For other symbols – see text

Mean O def during maximal exercise showed a minor decrease at 7 % COHb and a more marked (12 increase at 20 % COHb which was significant ($p < 0.05$) Mean peak HLa increased by 2 % at 7 % COHb and by 10 % at 20 % COHb None of these changes were significant

2) *Treadmill experiment* a In the 5 subjects in the first part of this study the attained COHb levels at each exposure levels were 7 1 % and 19 3 % respectively The effects of CO were of the same nature but more pronounced than the corresponding measurements on the bicycle ergometer Thus mean work time was lowered from 5 min 32 s on control days to 4 min 4 s at the 7 % COHb ($p < 0.01$) and to 2 min 54 s at 20 % COHb ($p < 0.001$) Mean max $\dot V_{O_2}$ decreased by 9 % at 7 % COHb and by 23 % at 20 % COHb Both changes were highly significant ($p < 0.01$) Mean max HR was lowered by 2 beats/min at 7 % COHb ($p > 0.1$)

Bicycle								Treadmill						
maximal load								maximal load						
COHb % sat	O D L	V_O L/min	HR Beats/min	V_E L/min	HLa mM	WL KPM	WT Min	COHb % sat	V_O L/min	HR Beats/min	V_E L/min	HLa mM	WL *	WT Min
NM	2 56	3 28	180	137 9	11 7	1620	3 66	NM	4 12	186	151 4	11 0	14/3	4 75
NM	2 84	3 63	167	118 2	7 8	1800	3 47	NM	4 15	175	148 8	12 9	15 3	4 83
NM	NM	4 19	180	127 5	15 6	1800	5 12	NM	4 92	188	164 8	16 5	15 3	6 75
NM	3 71	3 53	188	153 2	14 3	1800	4 42	NM	4 05	195	171 1	15 9	15 3	5 72
NM	2 99	3 56	205	111 0	12 9	1700	4 25	NM	4 35	210	144 5	16 2	15 3	5 12
	2 93	3 64	184	127 5	12 4	—	4 18	—	4 32	191	156 11	14 5	—	5 53
	0 27	0 34	14	19 7	3 0	—	0 65	—	0 36	13	11 98	2 4	—	0 80
73	2 03	3 05	180	134 4	11 3	1620	2 92	7 7	3 59	183	156 2	11 0	14 3	3 67
81	3 25	3 25	163	118 1	10 2	1800	2 33	6 0	3 86	175	144 4	11 4	15/3	3 83
80	NM	3 91	179	127 4	15 4	1800	4 47	7 7	4 63	180	189 0	14 6	15/3	5 00
70	3 41	3 17	186	182 2	12 9	1800	3 22	6 3	3 69	193	174 7	12 3	15/3	3 52
74	2 19	3 12	200	113 7	14 0	1700	3 23	7 7	3 83	212	139 8	17 6	15 3	4 33
75	2 98	3 30	182	135 15	12 4	—	3 23	7 1	3 92	189	160 62	13 4	—	4 06
—	0 54	0 35	13	27 51	2 0	—	0 78	—	0 41	15	20 40	2 6	—	0 60
20 0	3 20	2 56	179	124 6	11 9	1620	2 17	19 2	3 12	182	156 0	9 4	14/3	2 02
20 5	3 25	2 86	165	140 8	11 0	1800	1 83	19 6	3 27	168	150 3	11 4	15/3	2 33
2 2	NM	3 28	176	125 3	15 8	1800	3 30	20 3	3 86	173	185 1	18 5	15/	3 67
21 1	3 38	2 67	183	166 9	14 2	1800	2 17	18 1	2 95	189	183 8	15 5	15/3	2 53
20 6	3 18	2 88	201	114 4	15 6	1700	2 75	18 9	3 48	201	134 4	15 9	15/3	3 08
20 7	3 25	2 85	180	134 44	13 8	—	2 45	19 2	3 34	183	161 92	14 2		2 90
—	0 09	0 27	13	20 46	2 2	—	0 58	—	0 35	13	22 05	3 7	—	0 78

and 8 beats/min ($p < 0.05$) at the 20 % COHb No significant changes were observed in mean peak HLa and max V_E at both levels of exposure

b) The results from the experiments in the second part of this study where all the 10 subjects performed maximal treadmill exercise at 3 different levels of COHb varying between 4 8 % and 21 2 % are illustrated in Fig 1 Both work time on the fixed maximal work load and oxygen uptake at this work level decreased with increasing levels of COHb The correlation coefficients were 0 79 and 0 85 respectively

Discussion

In this study the circulatory effects of carbonmonoxide inhalation prior to submaximal and maximal exercise have been investigated

Maximal physical performance here characterized as maximal work time (MWT) during standard exhaustive exercise on the treadmill and the bicycle ergometer, was reduced with increasing levels of COHb It is well known that the maximal work time on a standard exhaustive work load is not an accurate index of the performance capacity its dependence on psychological factors in addition to physiological determinants complicates the evaluation of the noticed effects Nevertheless the significant correlation (r = 0 79) observed between the decrease in the work time on the treadmill and the amount of COHb (Fig 1) clearly illustrates the limiting effects of COHb even in low amount, on MWT The unusually large decrease in MWT (38 %) for a 6 3 % COHb level on Fig 1 is more than expected at this moderate reduction of the oxygen transporting capacity of the blood

One interesting observation in our study is that if the regression line (Fig 1) relating decrease in work time to % COHb is extrapolated to the right, work time should be reduced to zero at a COHb level of 46 % According to the established relationship between max V_{O} and COHb (see below and Fig 1) a 46 % COHb level should correspond to 40 % decrease in max V_{O_2} which should not reduce the performance time to zero On control days the subjects could run for an average of 6 min 32 s with a mean peak HLa level of 14 5 mM in the blood At 7 % COHb they ran only 4 min 4 s but the mean peak HLa level showed only a minor decrease At the 20 % COHb MWT was decreased to 2 min 54 s but the mean peak HLa level showed again only an unsignificant decrease One can thus see that with increasing COHb levels MWT gets shorter and shorter and seems to be associated with a given rather fixed concentration of HLa It can then be postulated that this amount of lactate or more precisely its equivalent in the working muscle (Karlsson 1971) would be produced almost instantly at a COHb level of 46 % and thus stop exercise in a very short time In this respect it is interesting to note that Roughton (1944) mentions that subjects at mild exercise or even at rest will collapse when their COHb is in the 40 to 50 % range

As mentioned above the peak concentration of blood HLa at the end of the maximal work at both levels of exposure did not vary significantly from control values This is expected since the absolute amount of HLa or factors related to the HLa production is probably what in many work situations limits the performance (Karlsson 1971) This end point is reached earlier as COHb increases The lactate production is apparently highly related to the oxygen deficit (O def) during work (Astrand and Rodahl 1970 Karlsson 1971) Assuming that the lactate production was similar in control experiment and in experiments after CO exposure respectively the variations in O def at maximal exercise are expected The O def is thus not increased by the CO but is attained faster than in control experiments

The reduced physical performance capacity can at least partly be explained by a decrease in max V_{O_2}, which decreased with increasing levels of COHb (r = 0 85) — see Fig 1 Pirney *et al* (1971) reported a reduction of max V_{O_2} of 15 % at a COHb concentration of 15 4 % Vogel *et al* (1971) report a 23 % decrease in max V_{O_2} after CO breathing but in their report they do not mention the COHb level (O

binds itself to Hb rendering it nonfunctional for O transport but CO administration will also shift the O dissociation curve of residual Hb the left (Roughton 1944) thus making tissue oxygen extraction more difficult Therefore one might expect a more marked decrease in max V_{O_2} than that actually observed at high COHb levels While no data on exercising muscles venous P_{O_2} are available Saltin et al (1968) reported femoral vein P_{O_2} of 12 mm Hg at maximal exercise At such low P_{O_2} tensions and for levels of COHb $\leqslant 20\%$ the left sided shift in the O dissociation curve induced by CO becomes negligible so that effect of CO intoxication would be related at the exercising muscular levels only to the decreased amount of functional Hb

The highest HR obtained during maximal work (max HR) compared to control studies decreased in average 8 beats/min during the treadmill run and 4 beats/min during the bicycle work at 20 % COHb both changes being significant This decrease in max HR could in part be explained by the decrease in MWT after CO loading The relation HR versus work time during the maximal exercise in the control and CO experiments are apart from the first min of exercise almost identical Thus at exhaustion during the treadmill experiments the max HR at 7 % COHb and 20 % COHb were 189 and 183 beats/min respectively The HR at corresponding work time in the control experiments when the subjects stopped during the CO experiments were 189 and 185 beats/min respectively and from this point the HR then increased to 191 beats/min at the end of the maximal exercise in the control experiments Piney *et al* (1971) and Vogel *et al* (1971) reported no significant change in max HR after CO exposure compared to control experiments

During submaximal work at both 7 % and 20 % COHb the heart rate was increased compared to control values and these increases were significant except during the 30 % work load with 7 % COHb A highly positive and significant correlation could be established between the heart rate increase and the amount of COHb at the heavy work load ($r = 0.87$—$p < 0.002$) The finding of an increased heart rate is in agreement with the data reported by other investigators (Asmussen and Chiodi 1941 Chiodi *et al* 1941 Asmussen and Knudsen 1943 Klausen *et al* 1968 Avnes *et al* 1969 Piney *et al* 1971 Vogel *et al* 1971) This increase in heart rate at comparable levels of work has apart from the reduced oxygen transport capacity been ascribed to a pressoregulatory compensation for a CO induced peripheral vasodilatation with secondary lower filling pressure of the heart and lower stroke volume (Asmussen and Chiodi 1941) and also to a compensation for a CO induced lowered blood volume (Asmussen and Knudsen 1943)

At submaximal exercise the O def reflects the degree of anaerobic metabolism at the beginning work Since at the 30 % work load both at 7 % and 20 % COHb there was no change in O def it is presumed that the work level was light enough so that the added burden of up to 20 % COHb did not affect the rate of rise of oxygen uptake up to steady state level That lactates at the termination of the work period at both 7 % COHb and 20 % were not elevated was more or less expected since oxygen uptake did not exceed 40 % of max V_O At high submaximal wor

here was no significant change in O def at 7 % COHb but at 20 % COHb there was a probably significant change ($p \leqslant 0.1$)

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Effect of Adrenaline on Amino Acid Transport in Perfused Rat Heart

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Abstract

Isaksson O and B Kallfelt *Effect of adrenaline on amino acid transport in perfused rat heart* Acta physiol scand 1972 86 483—497

Effects of adrenaline on amino acid transport in the isolated perfused rat heart was investigated using the non utilizable amino acids α aminoisobutyric acid (AIB) and 1 aminocyclopentane carboxylic acid (cycloleucine) Adrenaline added *in vitro* caused a dose dependent increase in the uptake of AIB and cycloleucine and a significant stimulation was seen after 15 min of perfusion The minimal effective concentration was 5×10^{-8} M and an effect was obtained when hearts were perfused with glucose or palmitate as substrate and when no exogenous substrate was added Adrenaline increased the uptake in hearts arrested with high [K⁺] or tetrodotoxin and the accelerated uptake was not dependent upon the chronotropic effect of adrenaline Under conditions when adrenaline increased amino acid uptake protein synthesis was either unaffected or decreased The data of the present study show that adrenaline directly stimulates amino acid transport in the heart and it is concluded that increased uptake of amino acids could be an energy generating system for the heart during adrenergic stimulation

Adrenaline is known to exert several important effects on the mechanical performance and on various biochemical activities of cardiac muscle The metabolic effects include increased glycogenolysis and lipolysis as well as increased oxygen consumption The glycogenolytic and lipolytic effects of adrenaline in heart muscle have been extensively studied and analysed There are on the other hand very few reports concerning the effects of adrenaline on the amino acid metabolism and protein synthesis in the heart *In vivo* administration of adrenaline is known to decrease the amino acid level of the plasma (Noall *et al* 1957) suggesting either an increased uptake or a decreased release of amino acids from the tissues In favour of the former hypothesis are the experiments of Sanders and Riggs (1967) in which injection of adrenaline was shown to enhance the uptake of the non utilizable amino acids α aminoisobutyric acid (AIB-^{3}H) and 1 aminocyclopentane carboxylic acid (cycloleucine ^{14}C) in the heart and liver of the rat It is however well known that adrenaline can cause the release of adrenocorticotrophic hormone (ACTH) leading to an increased secretion of glucocorticoids which can markedly influence protein metabolism in many organs It is also well established that adrenaline can inhibit

the release of insulin a hormone which has strong effects both on amino acid transport and protein synthesis in several tissues These facts complicate the interpretation of the increased uptake of AIB ^{3}H and cycloleucine ^{14}C found *in vivo* following adrenergic stimulation By using an isolated perfused rat heart preparation these difficulties can be overcome since secondary homeostatic mechanisms are excluded and the primary effect of adrenaline can be evaluated

In two preceding studies (Hjalmarson and Isaksson 1972, Ahren *et al* 197[illegible]) it was found that increased afterload stimulated the amino acid uptake and protein synthesis in the heart muscle It was therefore also of interest to investigate if another type of increased heart work—caused by addition of adrenaline—could produce the same pattern of changes on the membrane transport of amino acids and protein synthesis as increased afterload

In the present study the effects of adrenaline on amino acid transport in the perfused heart have been studied by using the non utilizable amino acids AIB ^{3}H and cycloleucine ^{14}C Incorporation of phenylalanine ^{3}H into heart protein has also been studied under various experimental conditions

Methods

Hearts were removed from fed male rats of the Sprague Dawley strain weighing 2[illegible]0–[illegible]0 g The rats were anesthetized with Nembutal (60 mg/kg b w) and the hearts were rapidly excised and transferred to ice chilled saline The aorta and the left atrium were cannulated and retrograde perfusion with Krebs Henseleit bicarbonate buffer at 37 C from a reservoir 70 cm above the heart was started immediately Following a 5 min retrograde wash out period the heart was either perfused as a modified Langendorff preparation (retrograde perfusion non working hearts Neely *et al* 1967) or as a working heart preparation (anterograde perfusion working hearts Morgan *et al* 1965 Neely *et al* 1967) The perfusion medium was Krebs-Henseleit bicarbonate buffer containing the disodium salt of EDTA (0 5 mM) with glucose (11 mM) or palmitate (1 [illegible] mM) as substrate When palmitate was used a fatty acid albumin complex was prepared as described previously (Hjalmarson and Isaksson 197[illegible]) In some experiments the perfusion medium contained all amino acids at normal rat plasma levels (Morgan *et al* 1971) The recirculating perfusion volume was 10 ml during the subsequent periods The temperature of the perfusate was maintained at 37 C (in 1 expt at 32 C as indicated by Table IV) and the buffer was continuously gassed with different gas mixtures equilibrated with water at 37 C [95 % O_2 5 % CO_2 (Control) 30 % O_2 65 % N_2 5 % CO_2 95 % N_2 5 % CO_2 (Hypoxia)] The perfusion pressure in retrogradely perfused hearts was 60 mm Hg Antrogradely perfused hearts were perfused at a left atrial filling pressure of 10 cm H_2O and the heart [illegible] pump the perfusate against a hydrostatic pressure head of 70 cm H_2O [illegible] and heart rate were recorded by a transducer attached to a manifold [illegible] and the pressure tracings were monitored by a Sanborn recorder 16-1300 S [illegible] measured by collecting the fluid dropping from the heart in a graduated [illegible]

To measure the oxygen te[illegible] effluent the pulmonary artery was cannulated with a plastic tube This [illegible] med after the cannulation of the aorta At regular time intervals (30 and 60 [illegible]) the tube was connected to a syringe and samples were collected by withdraw[illegible] sate by the syringe without exposure to air Samples were withdrawn from the [illegible] at the same time Oxygen tension of the perfusate samples were estimated with [illegible] ode (F 5046 Radiometer Copenhagen)

Adrenaline was dissolved in such a [illegible] Krebs-Henseleit bicarbonate buffer that addition of 100 μl of this solution at th[illegible] f the perfusion period gave the desired molarity for adrenaline in the final perfu[illegible]

Asystole was induced in retrogradely perf[illegible] hearts by increasing the potassium concentration to 34 meq/l (reducing the concentrat[illegible] Na) to maintain isotonicity) or by a[illegible] of tetrodotoxin (5 μg/ml) to the perfusate When retrogradely perfused hearts were electrically paced the right atrial tissue was exci[illegible] and the A—V nod[illegible] crushed to induce [illegible]

TABLE I Sucrose ¹⁴C spaces and total tissue water of isolated perfused rat heart

Experimental condition	Number of hearts	Sucrose ¹⁴C space	Total tissue water
Working			
Control	5	35 4 ± 0 8	81 3 ± 0 38
Adrenaline 10⁻⁶ M	5	34 3 ± 0 7	81 1 ± 0 3
Non-working			
Control	6	37 5 ± 0 9ᵃ	80 8 ± 0 37ᶠ
Perfusate gassed with 30 % O₂ 65 % N₂, 5 % CO₂	6	36 8 ± 0 9ᵇ	82 3 ± 0 46ᵍ
Hypoxia (perfusate gassed with 95 % N₂, 5 % CO₂)	6	40 6 ± 1 2ᶜ	84 2 ± 0 64ʰ
[K⁺] 34 meq/l	4	74 3 ± 4 2ᵈ	83 6 ± 1.20ⁱ
Tetrodotoxin 5 μg/ml	4	42 8 ± 1 8	84 4 ± 0 39ʲ
Adrenaline 10⁻⁶ M	6	30 8 ± 1 1	80 4 ± 0 41
Aminophylline 10⁻³ M	6	31 3 ± 0 8	81 2 ± 0 31

Hearts were perfused anterogradely (working) or retrogradely (non working) for 30 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) and sucrose ¹⁴C Values are means ± S E of per cent of wet tissue weight

a vs c d e $p < 0.05$ f vs h i j $p < 0.05$ a vs b NS f vs g NS

ventricular rhythm (approx 100 beats/min) Platinum electrodes were implanted in the right ventricle wall and the hearts were thereafter paced at different frequencies with an electronic stimulator (5 V at 1 ms) All hearts followed the artificial pacemaker throughout the perfusion period

Chemicals

Radioactive substances were obtained from New England Nuclear Co Boston USA The substances were used with the following specific activities and molarities α aminoisobutyric acid³H(G) (AIB-³H) 0 4 μCi/μmole 0 05 mM 1 aminocyclopentane 1-carboxylic acid-carboxyl ¹⁴C (cycloleucine ¹⁴C) 0 04 μCi/μmole 0 1 mM L-phenylalanine ³H (G) 1 25 μCi/μmole 0 08 mM sucrose ¹⁴C (U) 3 96 μCi/μmole 0 01 mM Adrenaline bitartrate was obtained from ACO Sweden Aminophylline [(Theophylline)₂ Ethylenediamine] and tetrodotoxin from Sigma USA

Determination of total tissue water and extracellular water

Total tissue water was determined by drying a piece of the lower part of the left ventricle to constant weight at 100 C in a vacuum oven The extracellular space of the perfused heart was determined by measurements of the distribution of sucrose ¹⁴C between the tissue and the perfusate The total tissue water and sucrose ¹⁴C space is expressed as per cent of the wet tissue weight (ml/100 g tissue) Table I shows the sucrose ¹⁴C space and total tissue water in working and non working hearts perfused for 30 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) under various experimental conditions The sucrose ¹⁴C space and total tissue water content were increased by hypoxia (perfusate gassed with 95 % N₂ and 5 % CO₂) or by perfusion with tetrodotoxin Perfusion with a high potassium concentration (34 meq/l) decreased the sucrose ¹⁴C space and increased the total tissue water of the heart Adrenaline (10⁻⁶ M) aminophylline (10⁻³ M) and gassing the perfusate with 30 % O₂ 65 % N₂ and 5 % CO₂ did not significantly affect the sucrose ¹⁴C space or total tissue water content

Determination of the intracellular accumulation of AIB-³H and cycloleucine ¹⁴C and incorporation of phenylalanine ³H

At the end of the perfusion period the hearts were cut in a beaker containing ice-chilled saline About 250 mg of the lateral wall of the left ventricle was excised rinsed rapidly in buffer weighed and homogenized in of 5 % perchloric acid (PCA) The intracellular accumulation of the substances is he distribution ratio of radioactivity between

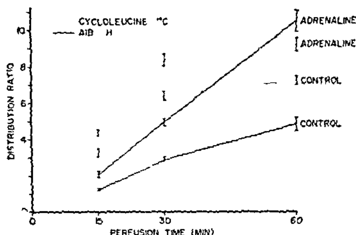


Fig 1 Effects of adrenaline (10^{-6} M) on the accumulation of AIB-^3H and cycloleucine ^{14}C by perfused working rat heart. Hearts were perfused for various periods with Krebs-Henseleit bicarbonate buffer containing glucose (11 mM) with and without adrenaline Each point represents a mean of 5—8 observations and standard errors are indicated by vertical lines The effect of adrenaline was significant at all time periods studied ($p < 0.05$)

intra and extracellular compartments (cpm/ml intracellular water cpm/ml medium) as described previously (Hjalmarson and Isaksson 1972) Liquid scintillation counting was carried out with 7 ml of Insta Gel (Packard) Double labelled samples were counted in a Tri Carb Packard Model 3375 spectrometer with settings which gave a 10—15 % overlap of ^{14}C radioactivity in the ^3H channel and less than 1 % overlap of ^3H activity in the ^{14}C channel The two isotopes (cycloleucine ^{14}C and AIB-^3H) were counted with an efficiency of about 20 % for ^3H and 60 % for ^{14}C The standard error was less than 2 % for the determination of sucrose ^{14}C AIB-^3H and cycloleucine ^{14}C The degree of quenching was [illegible]ed by automatic external standardization in each sample and was found to be the same for tissue extracts and their respective perfusion media It was therefore not necessary to correct the counting data for quenching prior to the calculations of the extracellular spaces and distribution ratios The incorporation of phenylalanine ^3H into heart protein was determined as described previously (Hjalmarson and Isaksson 1972) and is expressed as DPM/mg protein × 10^{-3}

Statist

Analysis of variance (with one criterion of classification) followed by the Student Newman Keuls multiple range test was used (Woolf 1968) A p-value of 0.05 or less was considered significant in this study

Results

Effects of adrenaline on the accumulation of AIB ^3H and cycloleucine ^{14}C

A time-course study of the effects of adrenaline (10^{-6} M) on the accumulation rate of AIB ^3H and cycloleucine ^{14}C in the perfused working rat heart was performed as shown by Fig 1 The figure clearly shows that adrenaline stimulated the uptake of AIB ^3H and cycloleucine ^{14}C For both AIB ^3H and cycloleucine ^{14}C a stimulatory effect was seen after only 15 min of perfusion As shown previously (Ahren *et al* 1972) the kinetics for the concentrative uptake of AIB and cycloleucine were quite different For cycloleucine there was a rapid initial uptake during the first 30 min of perfusion followed by a slower phase during the subsequent 30 min while the accumulation of AIB continued to increase at a near linear rate for the whole perfusion period To further characterize the stimulatory effect of adrenaline on the amino acid transport hearts were perfused anterogradely for 30 min in the presence of various concentrations of adrenaline (Fig 2) It can be

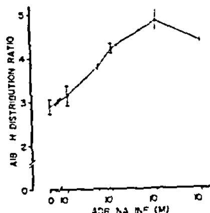


Fig 2 Effects of various concentrations of adrenaline on the accumulation of AIB-³H by perfused working rat heart. Hearts were perfused for 30 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) Each point represents a mean of 5—6 observations and the standard errors are indicated by vertical lines. A significant stimulatory effect of adrenaline was seen at a concentration of 5×10 M ($p < 0.05$)

seen from the figure that adrenaline caused a dose-dependent increase in the uptake of AIB-³H A significant stimulatory effect of adrenaline was seen at a concentration of 5×10⁸ M and a maximal effect at a concentration of 10⁶ M which indicates that the dose response curve was rather steep Adrenaline had no effect at a concentration of 10⁸ M At a concentration of 5×10⁸ M a significant elevation of the peak aortic systolic pressure was seen (not shown in the figure) but not at 10⁸ M indicating that there was a parallelism between the inotropic response and the stimulation of AIB-³H uptake

In order to investigate the effects of adrenaline on amino acid transport under conditions closer to an *in vivo* situation all amino acids at normal rat plasma levels

TABLE II Effects of adrenaline on accumulation of AIB- H and cycloleucine C of isolated rat heart.

Addition to perfusate	Perfusion period min	Distribution ratio		Control		Adrenaline 10⁻⁴ M
No addition	30	AIB- H		5 36 ± 0 29 (4)	p < 0 05	6 80 ± 0 31 (4)
		Cycloleucine	C	7 41 ± 0 34 (4)	p < 0 05	8 52 ± 0 12 (4)
Glucose 14 mM	30	AIB- H		2 66 ± 0 16 (5)[b]		5 16 ± 0 21 (5)[a]
		Cycloleucine	C	6 58 ± 0 32 (5)		8 41 ± 0 26 (6)
Albumin (3) + palmitate 1 5 mM	60	AIB- H		3 17 ± 0 24 (5)		7 72 ± 0 43 (5)
		Cycloleucine	C	6 07 ± 0 28 (5)		7 46 ± 0 29 (5)
1×AA + glucose (14 mM)	60	AIB- H		1 57 ± 0 11 (6)		3 91 ± 0 18 (6)
		Cycloleucine	C	1 90 ± 0 08 (6)		2 63 ± 0 12 (6)

Hearts were perfused anterogradely (working) for 30 and 60 min with Krebs Henseleit bicarbonate buffer containing no substrate glucose (14 mM) albumin (3) palmitate (1 5 mM) or 1×AA (all amino acids at normal rat plasma levels) and glucose (14 mM) as indicated by the table Values are means ± S E Number of hearts in each group indicated in parentheses

a b $p < 0.05$ c and d $p < 0.05$

TABLE III Effects of adrenaline and aminophylline on accumulation of AIB-^{3}H and cycloleucine-^{14}C, heart rate and coronary flow of isolated rat heart

Addition to perfusate	AIB-^{3}H distribution ratio	Cycloleucine ^{14}C distribution ratio	Heart rate beats/min	Coronary flow ml g d w ×min
Control	2 19 ± 0 19^a	5 40 ± 0 28^a	231 ± 10	70 ± 3
Adrenaline 10 M	2 97 ± 0 12^b	7 15 ± 0 32^b	298 ± 5	85 ± 4
Aminophylline 10 M	2 4? ± 0 2?	5 9? ± 0 41^c	30? ± 7	87 ± 3
Adrenaline 10 M + Aminophylline 10 M	3 23 ± 0 17^d	7 29 ± 0 44^d	3?4 ± 9	89 ± 4

Hearts were perfused retrogradely for 30 min with Krebs Henseleit bicarbonate buffer containing glucose 14 mM) Values are means ± S E Six hearts in each group were perfused

a r b p 0 05 a rs d p 0 05 a rs c NS b rs d NS

or palmitate were added to the perfusion medium as shown by Table II It can be seen from the table that *adrenaline (10^{-6} M) accelerated the uptake of* AIB ^{3}H and cycloleucine ^{14}C in hearts which were anterogradely perfused for 60 min both when all amino acids at normal rat plasma levels or palmitate were added to the perfusion medium It is further seen from the table that adrenaline increased the distribution ratios of AIB ^{3}H and cycloleucine ^{14}C when hearts were perfused anterogradely for 30 min in the absence of glucose and that the distribution ratios these non utilizable amino acids were increased when glucose was absent in the on medium Thus a stimulatory effect of adrenaline on *the amino acid sport could be demonstrated* in the absence of glucose in *the presence of palmi* e and when the normal amino acid pattern in the plasma was simulated

It has been shown by several investigators that there is a rapid increase in the cardiac content of cyclic 3 5 AMP after administration of adrenaline Methyl xanthines which inhibit the phosphodiesterase enzyme and thereby the degradation of cyclic 3 5 AMP Butcher and Sutherland 1962) can increase the cardiac content of this nucleotide Although methylxanthines may also have other effects it was of interest to investigate if these substances like adrenaline could stimulate the *amino acid uptake in the perfused heart Table III shows an experiment in which* hearts were perfused in the presence of adrenaline (10^{-6} M) aminophylline (10^{-3} M) and adrenaline and aminophylline in combination The hearts were retrogradely perfused for 30 min Aminophylline increased the coronary flow and *heart rate to* the same extent as adrenaline but did not accelerate the uptake of AIB-^{3}H or cycloleucine ^{14}C No significant potentiation of the stimulatory effect of adrenaline on the uptake of the non utilizable amino acids by aminophylline could be registered either

Effects of adrenaline on the accumulation of AIB ^{3}H and cycloleucine ^{14}C in asystolic hearts

It has been *proposed that the metabolic* effects of adrenaline on the heart under certain conditions can be dissociated from the inotropic effects (Williamson and

[illegible] IV Effects of adrenaline on accumulation of AIB-³H and cycloleucine-¹⁴C, heart rate and coronary flow of arrested perfused hearts

[illegible]	Number of hearts	Adrenaline M	Distribution ratio: Cycloleucine-¹⁴C		Distribution ratio: AIB-³H		Heart rate beats/min	Coronary flow ml/g d w/min	
[illegible]	4	—			2.11 ± 0.14	p < 0.05	230 ± 7	63 ± 6	p < 0.05
	4	10^{-6}			2.76 ± 0.12		291 ± 6	85 ± 4	
[illegible]	4	—			0.28 ± 0.10	p < 0.05	0	78 ± 8	N S
	4	10^{-6}			1.18 ± 0.23		27 ± 5	46 ± 3	
[illegible]	4	—			2.12 ± 0.22	p < 0.05	sporadic contractions	61 ± 4	
	4	10^{-6}			3.05 ± 0.13		115 ± 6	69 ± 8	
[illegible] C	6	—	2.87 ± 0.15	p < 0.05	0.64 ± 0.10	p < 0.05	0	31 ± 4	p < 0.05
	6	5×10^{-7}	3.83 ± 0.27		1.01 ± 0.03		sporadic contractions	45 ± 3	

[illegible] a retrograde washout period of 5 min with Krebs-Henseleit bicarbonate buffer, hearts were [illegible]gradely perfused for 30 min in absence and presence of adrenaline. Cardiac arrest was induced [illegible] recirculation was started by addition of tetrodotoxin (5 μg/ml) to Krebs-Henseleit bicarbonate [illegible] or by increasing the potassium concentration in the buffer to 34 meq/l. The recirculating [illegible]sate contained glucose (14 mM). Values are means ± S.E.

[illegible]meson 1965; Hauge and Øye 1966). To elucidate if the increased uptake of [A]IB-³H and cycloleucine-¹⁴C caused by addition of adrenaline could be elicited [wi]thout chronotropic and inotropic effects, hearts were arrested by increasing the [pot]assium concentration in the perfusate to 34 meq/l or by addition of tetrodotoxin [(5] μg/ml) to the perfusion medium as shown by Table IV. In hearts perfused [ret]rogradely for 30 min under control conditions, adrenaline increased the AIB-³H [up]take, the heart rate and the coronary flow. Adrenaline (10^{-6} M) was also [fou]nd to increase the uptake of AIB-³H in hearts arrested with high potassium [con]centration or tetrodotoxin. However, adrenaline induced contractions in these [arr]ested hearts (Table IV) and it can therefore not be excluded that the acceler[ati]on of the AIB-³H uptake could be secondary to an increase in mechanical [act]ivity provoked by adrenaline. To overcome these difficulties the concentration [o]f adrenaline was decreased to 5×10^{-7} M and the temperature of the perfusate [w]as lowered to 32° C. Under these conditions adrenaline has been reported to be [wit]hout effect on the mechanical activity of the perfused heart (Hauge and Øye [19]66). As can be seen from Table IV adrenaline still stimulated the uptake of [A]IB-³H, and the uptake of cyclolecucine-¹⁴C was also stimulated, which suggests [th]at the increased amino acid uptake provoked by adrenaline can be dissociated [fr]om the increase in mechanical activity. However, sporadic contractions could [occ]asionally be seen some time after the addition of adrenaline in hearts perfused at [3]2° C, a fact that makes the interpretation of the results difficult. The accumula[t]ion of AIB-³H and the coronary flow were markedly decreased in hearts arrested

TABLE III Effects of adrenaline and aminophylline on accumulation of AIB-^{3}H and cycloleucine ^{14}C, heart rate and coronary flow of isolated rat heart

Addition to perfusate	AIB-^{3}H distribution ratio	Cycloleucine ^{14}C distribution ratio	Heart rate beats/min	Coronary flow ml/g d w × min
Control	2 19±0 19	5 40±0 28	231±10	70±3
Adrenaline 10^{-6} M	2 97±0 12^b	7 15±0 32^b	298±5	85±4
Aminophylline 10^{-3} M	2 42±0 24^c	5 94±0 41^c	302±7	87±3
Adrenaline 10^{-6} M+ Aminophylline 10^{-3} M	3 23±0 17^d	7 29±0 44^d	324±9	89±4

Hearts were perfused retrogradely for 30 min with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) Values are means±S E Six hearts in each group were perfused

a vs b $p < 0\ 05$ a vs d $p < 0\ 05$ a vs c NS b vs d NS

or palmitate were added to the perfusion medium as shown by Table II It can be seen from the table that adrenaline (10^{-6} M) accelerated the uptake of AIB ^{3}H and cycloleucine ^{14}C in hearts which were anterogradely perfused for 60 min both when all amino acids at normal rat plasma levels or palmitate were added to the perfusion medium It is further seen from the table that adrenaline increased the distribution ratios of AIB ^{3}H and cycloleucine ^{14}C when hearts were perfused anterogradely for 30 min in the absence of glucose and that the distribution ratios of these non utilizable amino acids were increased when glucose was absent in the perfusion medium Thus a stimulatory effect of adrenaline on the amino acid transport could be demonstrated in the absence of glucose in the presence of palmitate and when the normal amino acid pattern in the plasma was simulated

It has been shown by several investigators that there is a rapid increase in the cardiac content of cyclic 3 5 AMP after administration of adrenaline Methyl xanthines which inhibit the phosphodiesterase enzyme and thereby the degradation of cyclic 3 5 AMP (Butcher and Sutherland 1962) can increase the cardiac content of this nucleotide Although methylxanthines may also have other effects it was of interest to investigate if these substances like adrenaline could stimulate the amino acid uptake in the perfused heart Table III shows an experiment in which hearts were perfused in the presence of adrenaline (10^{-6} M) aminophylline (10^{-3} M) and adrenaline and aminophylline in combination The hearts were retrogradely perfused for 30 min Aminophylline increased the coronary flow and heart rate to the same extent as adrenaline but did not accelerate the uptake of AIB-^{3}H or cycloleucine ^{14}C No significant potentiation of the stimulatory effect of adrenaline on the uptake of the non utilizable amino acids by aminophylline could be registered either

Effects of adrenaline on the accumulation of AIB ^{3}H and cycloleucine ^{14}C in asystolic hearts

It has been proposed that the metabolic effects of adrenaline on the heart under certain conditions can be dissociated from the inotropic effects (Williamson and

TABLE IV Effects of adrenaline on accumulation of AIB-³H and cycloleucine ¹⁴C heart rate and coronary flow of arrested perfused hearts

Experimental condition	Number of hearts	Adrenaline M	Distribution ratio Cycloleucine ¹⁴C		Distribution ratio AIB-³H		Heart rate beats/min	Coronary flow ml/g d w min	
Control	4	—			2 11±0 14	p < 0 05	230±7	63±6	p < 0 05
	4	10^{-6}			2 76±0 1?		294±(	85±4	
[K+] 34 meq/l	4	—			0 28±0 10	p < 0 05	0	28±8	NS
	4	10^{-6}			1 18±0 23		27±5	46±3	
Tetrodotoxin 5 µg/ml	4	—			? 12±0 22	p < 0 05	sporadic contractions	61±4	
	4	10^{-6}			3 05±0 13		115±6	69±8	
[K+] 34 meq/l perfusate temp 32° C	6	—	? 87±0 15	p < 0 05	0 64±0 10	p < 0 05	0	31±4	p < 0 05
	6	5×10	3 83±0 2?		1 01±0 03		sporadic contractions	45±3	

After a retrograde washout period of 5 min with Krebs Henseleit bicarbonate buffer hearts were retrogradely perfused for 30 min in absence and presence of adrenaline Cardiac arrest was induced when circulation was started by addition of tetrodotoxin (5 µg/ml) to Krebs Henseleit bicarbonate buffer or by increasing the potassium concentration in the buffer to 34 meq/l The recirculating perfusate contained glucose (14 mM) Values are means ± S E

Jamieson 1965 Hauge and Øye 1966) To elucidate if the increased uptake of AIB-³H and cycloleucine ¹⁴C caused by addition of adrenaline could be elicited without chronotropic and inotropic effects hearts were arrested by increasing the potassium concentration in the perfusate to 34 meq/l or by addition of tetrodotoxin (5 µg/ml) to the perfusion medium as shown by Table IV In hearts perfused retrogradely for 30 min under control conditions adrenaline increased the AIB ³H uptake the heart rate and the coronary flow Adrenaline (10^{-6} M) was also found to increase the uptake of AIB ³H in hearts arrested with high potassium concentration or tetrodotoxin However adrenaline induced contractions in these arrested hearts (Table IV) and it can therefore not be excluded that the acceleration of the AIB ³H uptake could be secondary to an increase in mechanical activity provoked by adrenaline To overcome these difficulties the concentration of adrenaline was decreased to 5×10^{-7} M and the temperature of the perfusate was lowered to 32° C Under these conditions adrenaline has been reported to be without effect on the mechanical activity of the perfused heart (Hauge and Øye 1966) As can be seen from Table IV adrenaline still stimulated the uptake of AIB-³H and the uptake of cycloleucine ¹⁴C was also stimulated which suggests that the increased amino acid uptake provoked by adrenaline can be dissociated from the increase in mechanical activity However sporadic contractions could occasionally be seen some time after the addition of adrenaline in hearts perfused at 32° C a fact that makes the interpretation of the results difficult The accumulation of AIB ³H and the coronary flow were markedly decreased in hearts arreste

TABLE V Effects of different pacing frequencies on accumulation of AIB-³H and cycloleucine-¹⁴C of perfused rat heart

Parameter	Pacing frequency (imp/min) 125		250		375
AIB-³H distribution ratio	4.11 ± 0.20 (5)	NS	4.35 ± 0.19 (6)	NS	3.75 ± 0.31 (6)
Cycloleucine-¹⁴C distribution ratio	7.39 ± 0.44 (5)	NS	8.43 ± 0.30 (6)	NS	7.5[illegible] ± 0.38 (6)

Hearts paced (5 V at 1 ms) at different frequencies were retrogradely perfused for 60 min. By Krebs-Henseleit bicarbonate buffer containing glucose (14 mM). Values are means ± S.E. Number of hearts in each group is indicated in parentheses

with high potassium concentration in contrast to control hearts and hearts arrested with tetrodotoxin. This finding in combination with the decrease in sucrose-¹⁴C space (Table I) that was observed in hearts arrested with high potassium concentration raises the question of to what extent the functional integrity was preserved in these hearts.

Effects of different pacing frequencies on the accumulation of AIB-³H and cycloleucine-¹⁴C

The possibility that the rate of heart contractions *per se* was a determinant for the rate of amino acid uptake was tested by electrically pacing hearts at different frequencies as shown by Table V. Retrogradely perfused hearts were paced at 125, 250 and 375 impulses per minute and the uptake of AIB-³H and cycloleucine-¹⁴C was determined after a perfusion period of 60 min. It can be seen from the table that the accumulation of AIB-³H and cycloleucine-¹⁴C were not changed when the heart rate was varied between 125 and 375 imp/min. It is further seen from Table IV that the uptake of AIB-³H was not depressed in hearts arrested with tetrodotoxin. These observations exclude the possibility that the stimulatory effect of adrenaline on amino acid uptake was secondary to the chronotropic effect of adrenaline and the data further indicate that the rate of amino acid uptake is not directly linked to the rate of heart contractions.

Effects of reduced oxygen tension on the distribution of AIB-³H and cycloleucine-¹⁴C

Adrenaline increases the oxygen consumption of the heart. Since it has been reported that anoxia under certain conditions can increase the uptake of AIB-³H in the perfused rat heart (Jefferson *et al.* 1971), experiments were performed to delineate the eventual role of an oxygen deficit for the stimulatory effect of adrenaline on the uptake of AIB-³H and cycloleucine-¹⁴C. Table VI shows a time-course study of the effects of reduced oxygen tension on the distribution ratios of AIB-³H and cycloleucine-¹⁴C in retrogradely perfused hearts. Gassing the perfusate with 30 %

Table VI Effects of reduced oxygen tension on accumulation of AIB-^{3}H and cycloleucine-^{14}C in perfused rat heart

Parameter	Period of perfusion min	Perfusate gassed with 95% O_2 5% CO_2 (Control)	30% O_2 65% N_2 5% CO_2	95% N_2 5% CO_2 (Hypoxia)
AIB-^{3}H distribution ratio	15	1.19±0.07 (6)	1.11±0.07 (6)	1.21±0.10 (6)
	30	1.93±0.16 (6)	1.83±0.12 (6)	2.11±0.10 (9)
	60	3.30±0.26 (6)	3.30±0.11 (6)	4.37±0.23 (9)[b]
Cycloleucine-^{14}C distribution ratio	15	3.16±0.18 (6)	3.03±0.15 (6)	3.46±0.38 (6)
	30	6.31±0.34 (7)[c]	6.30±0.21 (6)	7.01±0.25 (8)[d]
	60	7.06±0.26 (6)	6.70±0.21 (6)	7.68±0.30 (6)[f]

Hearts were perfused retrogradely for various periods with Krebs-Henseleit bicarbonate buffer containing glucose (14 mM) gassed with different gas mixtures. Values are means±S.E. Number of hearts in each group is indicated in parentheses.

a vs b $p < 0.05$; c vs d NS; e vs f $p < 0.05$

O_2 65% N_2 and 5% CO_2 did not change the distribution ratios of AIB-^{3}H or cycloleucine-^{14}C at any of the perfusion periods studied (15, 30 and 60 min). By gassing the perfusate with 95% N_2 and 5% CO_2 (referred to as hypoxia) there was an increased distribution ratio of AIB-^{3}H and cycloleucine-^{14}C in hearts perfused retrogradely for 60 min confirming the results of Jefferson *et al.* (1971).

At 15 and 30 min of perfusion, however, the distribution ratios of AIB-^{3}H and cycloleucine-^{14}C were not increased by hypoxia. To find if the effects of adrenaline on amino acid transport could be due to hypoxia, the oxygen tension in the coronary effluent in retrogradely perfused hearts was directly measured. The perfusate was gassed with 95% O_2 and 5% CO_2 or 30% O_2, 65% N_2 and 5% CO_2 and the hearts were perfused for 60 min. As expected, adrenaline (10^{-6} M) accelerated the uptake of AIB-^{3}H and cycloleucine-^{14}C (perfusate gassed with 95% O_2 and 5% CO_2), while gassing the perfusate with 30% O_2, 65% N_2 and 5% CO_2 did not affect the distribution ratios of these amino acids (Table VII). It is further seen from Table VII that the oxygen tension was decreased in the coronary effluent of hearts perfused in presence of adrenaline, but the oxygen tension was lower in hearts *in which the perfusate was gassed with* 30% O_2, 65% N_2 *and* 5% CO_2. The coronary flow was increased by adrenaline and by gassing with reduced oxygen tension. The increase was more pronounced in hearts with reduced oxygen tension indicating that the coronary vessels were not maximally dilated in hearts perfused with adrenaline. It is also seen from Table VII that the heart rate was not significantly decreased in hearts in which the perfusate was gassed with a reduced oxygen tension. Since a decrease in the heart rate of the isolated perfused heart is one of the first effects following an oxygen deficit, the hearts seemed to tolerate an oxygen tension in the coronary effluent of approximately 50 mm Hg

TABLE VII Effects of adrenaline and reduced oxygen tension on accumulation of AIB-H and cycloleucine C in perfused rat heart

Parameter	Perfusate gassed with 95% O 5% CO₂		Perfusate gassed with 30 O 65% N 5 CO
	Control	Adrenaline 10 M	
AIB H distribution ratio	$3\,55 \pm 0\,20$	$5\,07 \pm 0\,28$	$3\,64 \pm 0\,24$
Cycloleucine C distribution ratio	$6\,94 \pm 0\,27$	$8\,69 \pm 0\,41$	$7\,18 \pm 0\,30$
Heart rate beats/min	230 ± 5	333 ± 22	218 ±8
Coronary flow ml g d w min	68 ± 2	88 ± 3	107 ± 5
Effluent O partial pressure (mm Hg)	125 ± 5	88 ± 4	47 ±[illegible]

Hearts were perfused retrogradely for 60 min with Krebs Henseleit bicarbonate buffer containing glucose (14 mM) Measurements of heart rate coronary flow and effluent O partial pressure were carried out at 30 and 60 min and means for individual hearts were calculated The O partial pressure in the aortic perfusate for the different groups was (mm Hg) Control $3?8 \pm 10$ Adrenaline 333 ± 8 Hearts perfused with reduced oxygen tension 149 ± 2 Values given in the table are means S E of six hearts

ell These results strongly suggest that adrenaline specifically increases the membrane transport of amino acids in the perfused heart and that the effects are not mediated by hypoxia

Effects of adrenaline on the incorporation of phenylalanine ³H in the anterogradely perfused heart

In general the rate of amino acid transport appears to be closely correlated to the rate of protein synthesis in several tissues It was therefore of interest to study if adrenaline could accelerate the incorporation of phenylalanine ³H into heart protein The effect of adrenaline on phenylalanine ³H incorporation was studied in hearts perfused anterogradely for 30 min in absence and presence of glucose (14 and 25 mM) It can be seen from Table VIII that adrenaline (10⁶ M) significantly decreased the incorporation rate of phenylalanine ³H when glucose was absent in the perfusate When glucose (14 or 25) mM was added to the perfusion medium adrenaline did not change the incorporation rate of phenylalanine ³H It is further seen from Table VIII that the incorporation rate of phenylalanine ³H was decreased when glucose was omitted from the perfusion medium Under several different experimental conditions adrenaline was found not to affect or decrease the incorporation rate of phenylalanine ³H into heart protein (unpublished observations) The fact that amino acid transport was stimulated by adrenaline under all experimental conditions studied suggests that the increase in the amino acid uptake evoked by adrenaline was *not directly linked to an acceleration of protein synthesis*

TABLE VIII Effects of adrenaline on incorporation of phenylalanine ^3H into heart protein of perfused rat heart

Glucose concentration in the perfusate mM	Phenylalanine ^3H incorporation rate DPM/mg protein × 10⁻		
	Control		Adrenaline 10^{-6} M
0	63±4 (6)	$p < 0.05$	48±2 (6)
14	104±5 (8)		101±3 (9)
28	10[illegible]±4 (6)		104±5 (5)

Hearts were retrogradely perfused for 30 min with Krebs-Henseleit bicarbonate buffer containing various concentrations of glucose in absence and presence of adrenaline (10^{-6} M) Values are means±S E Number of hearts in each group is indicated in parentheses.

Discussion

The ability of adrenaline to increase blood sugar is one of the most studied and longest known metabolic effects of adrenaline In contrast to the blood sugar increasing effect of adrenaline there is a decrease in the amino acid levels of the plasma subsequent to administration of adrenaline (Brunish and Luck 1952 Ellis 1956 Noall *et al* 1957) Part of this decrease can be explained by an accelerated gluconeogenesis in the liver following glycogen depletion provoked by adrenaline (Mallette *et al* 1969) but an increased uptake of amino acids in other tissues could also be of importance

In the present study the effects of adrenaline on the amino acid transport in the perfused rat heart have been investigated By using an *in vitro* preparation compensatory homeostatic mechanisms are excluded This is important when studying effects of adrenaline since it has been much discussed whether the decreased amino acid levels following adrenaline administration *in vivo* is mediated by insulin secreted in response to hyperglycemia (for review see Russell 1955) In the present study adrenaline was found to increase the amino acid uptake in the perfused rat heart after a perfusion period of only 15 min The amino acid uptake was accelerated in the absence of exogenous substrates and when all amino acids at normal plasma levels were added to the perfusate (Table II) The fact that adrenaline stimulated the uptake of AIB and cycloleucine in the absence of glucose and in the presence of palmitate excludes the possibility that the increased uptake was secondary to an accelerated glucose uptake by the heart which is known to occur following adrenergic stimulation (Williamson 1964) A significant stimulatory effect could be registered at a concentration of 5×10^{-8} but not at 10^{-8} M of adrenaline and a clear dose response relationship was evident when the concentration was increased from 5×10^{-8} to 10^{-6} M These observations demonstrate that adrenaline can directly stimulate the amino acid uptake of the heart confirming the conclusion reached by Sanders and Riggs (1967) from *in vivo* studies in the rat There seems to be important differences between the heart and the skeletal muscles concerning t metabolic effects of adrenaline Whereas adrenaline increases the amino acid u (Sanders and Rigg 1967 results from the present tudy) and the glu

(Williamson 1964) in the heart, adrenaline does not affect the amino acid uptake (Noall *et al* 1957 Eichhorn *et al* 1961, Riggs 1964) in skeletal muscles and has no clear effect on the glucose uptake of skeletal muscles (Ellis 1956)

In a previous study (Ahrén *et al* 1972) it was shown that increased pressure load on the heart resulted in an accelerated uptake of AIB and cycloleucine Since adrenaline increased the mechanical activity of the heart when an acceleration of the uptake of AIB and cycloleucine was observed it was of interest to study the effects of adrenaline in the absence of inotropic and chronotropic effects For this purpose the hearts were arrested by perfusion with high potassium concentration or by adding tetrodotoxin to the perfusion medium High $[K^+]$ abolishes spontaneous contraction by depressing pacemaker automaticity Tetrodotoxin inhibits the mechanical activity by blocking the large increase in sodium permeability that is associated with membrane depolarization (Narahashi *et al* 1960) Sporadic contractions were seen at the end of the perfusion period of 30 min in hearts arrested with tetrodotoxin probably due to the fact that the concentration of tetrodotoxin (5 μg/ml) used was too low for complete cardiac arrest Adrenaline at a concentration of 10^{-6} M induced regular contractions in hearts perfused with tetrodotoxin and high $[K^+]$ at 37° C which complicates the interpretation of the increased AIB uptake found in the presence of adrenaline under these circumstances However when the temperature of the perfusate was lowered to 32° C and the concentration of adrenaline decreased to 5×10 [illegible] M adrenaline still increased the uptake of AIB and cycloleucine These results indicate that the effect of adrenaline on the amino acid transport is not directly related to the increase in mechanical activity In this connection it is of interest to note that adrenaline has been reported to increase the oxygen consumption of hearts arrested with high $[K^+]$ (Challoner and Steinberg 1965 Hauge and Øye 1966) Adrenaline has also been found to increase the amount of phosphorylase *a* and to exert a lipolytic effect in asystolic hearts (Øye 1967 Challoner and Steinberg 1965) and it is suggested from the results of the present study that an increased uptake of amino acids is a primary metabolic effect of adrenaline It must however be emphasized that this does not exclude the possibility that the increased mechanical activity caused by adrenaline also was a determinant for the acceleration of amino acid transport In general the per cent increase in the distribution ratios of AIB ^{3}H and cycloleucine ^{14}C provoked by adrenaline was larger in the anterogradely perfused (working) hearts compared to the retrogradely perfused (non working hearts (*cf* Table II and III) Since the external heart work as indicated by a larger increase in aortic pressures and coronary flow was proportionally more increased by adrenaline in working hearts the more pronounced effect of adrenaline on the amino acid transport in working hearts could have been due to mechanical factors An increase in heart rate leads to an accelerated uptake of $[Ca^{2+}]$ by cardiac cells and under most conditions to an increased force of contraction (Blinks and Koch Weser 1961 Shelburne *et al* 1967) Since the same principal adjustments occur after administration of adrenaline an increase in heart rate can to some extent simulate the effects of adrenaline The finding that the distribution

ratios of AIB and cycloleucine were not changed when the heart rate was increased from 195 to 375 beats/min by electrical pacing practically rules out the possibility that the uptake of AIB and cycloleucine was directly dependent upon the chronotropic effect of adrenaline The conclusion that the heart rate was not a major determinant for the rate of amino acid uptake was also supported by the observation that the distribution ratio of AIB was not at all affected when hearts were arrested with tetrodotoxin (Table IV) The observation of Jefferson *et al* (1971) that the protein synthesis was not affected when perfused hearts were arrested with tetrodotoxin is a further example of a dissociation of metabolic processes from contractile events in the heart

It is well known that adrenaline increases the oxygen consumption of the heart In the present study the oxygen tension in the coronary effluent was decreased from approx 175 to approx 90 mm Hg when hearts were retrogradely perfused in the presence of adrenaline The possibility that the stimulatory effect of adrenaline on the amino acid transport was secondary to a decreased supply of oxygen to the heart was practically ruled out by the experiment presented in Table VII In this experiment the oxygen tension in the coronary effluent of hearts gassed with a reduced oxygen tension (30 % O_2 65 % N_2 5 % CO_2) was consistently lower than in hearts perfused with adrenaline Yet no stimulation of amino acid transport was evident in these hearts It has been reported that noradrenaline is released from nerve terminals in isolated rat hearts during hypoxic conditions (Wollenberger and Shahab 1965) Although the effects of noradrenaline have not been investigated in the present study it is possible that the increase in the distribution ratio of AIB during hypoxic conditions could have been due to a stimulatory effect of noradrenaline In the present study hypoxia (perfusate gassed with 95 % N_2 and 5 % CO_2) increased the distribution ratio of AIB after 60 min of perfusion confirming the results of Jefferson *et al* (1971) It was also reported in the same study that hypoxia markedly decreased protein synthesis The unexpected finding that hypoxia increased the distribution ratio of AIB and cycloleucine after 60 min of perfusion is difficult to explain since it is postulated that the membrane transport of amino acids is an energy requiring process (for ref see Christensen 1962)

It is now generally accepted that the glycogenolytic and lipolytic effects of the catecholamines can be traced back to an increased production of cyclic 3 5 AMP (Sutherland *et al* 1968) The possibility that the increased membrane transport of amino acids caused by adrenaline was directly dependent upon an elevated cardiac content of this nucleotide has not been completely investigated in the present study The failure of aminophylline to increase the distribution ratios of AIB and cycloleucine and to potentiate the effects of adrenaline may be an argument against a direct involvement of cyclic 3 5 AMP for the acceleration of amino acid transport However no dose response experiments were performed with aminophylline thus the absence of effect could have been due to a too high or a too low concentration of the methylxantinederivate The hypothetical involvement of cyclic 3 5 AMP for the increase in amino acid uptake caused by ad

must await further experiments including direct measurements of the nucleotide in the cardiac tissue during different experimental conditions

Increased uptake of amino acids in muscle tissue is generally associated with an increased protein synthesis (Christensen 1962 Riggs 1964) Under no circumstances however was adrenaline found to increase the incorporation rate of phenylalanine ^{3}H into heart tissue protein The results presented in Table VIII show that adrenaline did not affect the incorporation rate of phenylalanine ^{3}H when glucose was included in the perfusion medium and that adrenaline decreased the incorporation of phenylalanine ^{3}H when glucose was absent

Further adrenaline has been shown to decrease the incorporation of amino acids into tissue proteins when added *in vitro* to the cut diaphragm preparation (Wool 1960) to rabbit liver slices (Pryor and Berthet 1960) and to epididymal adipose tissue (Herrera and Renold 1960) Thus from the results of the present study and from earlier investigations using different tissues in other laboratories it is evident that adrenaline decreases the rate of protein synthesis at least under certain conditions Since the incorporation of amino acids into tissue protein is an energy requiring process an inhibition of the protein synthesis provoked by adrenaline would supply more energy for the contractile processes in the heart under periods of cardiac stress The increased uptake of amino acids by the heart during adrenergic *stimulation could also be of importance for generation of energy It has been postu*lated that amino acids can be catabolized in skeletal muscles through an "alanine cycle" (Mallette *et al* 1969) In this cycle alanine is formed by amino transfer to pyruvate from any of several amino acids This cycle provides a means by which amino acids can be metabolized in extrahepatic tissues without release of the toxic metabolite ammonia (Mallette *et al* 1969) Alanine is the only amino acid put out by the heart *in vivo* (Carlsten *et al* 1966 Carlsten *et al* 1967) which suggests that amino acids also can be catabolized by means of this cycle in the heart Thus the increased uptake of amino acids evoked by adrenaline could be an important energy generating system for the heart during adrenergic stimulation

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Steady State Contribution of the Na, K-Pump to the Membrane Potential in Identified Neurons of a Terrestrial Snail, Helix pomatia

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Abstract

CHRISTOFFERSEN G R J *Steady state contribution of the Na K pump to the membrane potential in identified neurons of a terrestrial snail, Helix pomatia* Acta physiol scand 1972 86 498—514

The presence of an electrogenic Na K pump has been demonstrated in identified neurons of the snail *Helix pomatia* by the observation of ouabain sensitive hyperpolarizations following increase of intracellular sodium concentration A contribution of the pump to the resting membrane potential (RMP) has been measured at varying extracellular potassium concentration as the depolarizing effect of ouabain upon RMP The contribution was found to have a value of −7 mV at 4 mM external potassium and to decline with increasing and decreasing K concentrations The electrogenic flux has been calculated by two independent methods from measurements of membrane resistance and the electrogenic contribution to the RMP Agreement was found when the flux was computed both from modified constant field equations and from Ohm s law The flux increased from zero at 0 mM external potassium concentration to −10 peq cm s ⁻¹ at 6 mM and stayed at this level with rising concentrations of K It is concluded that the decrease of the electrogenic contribution to RMP observed at low K concentrations is caused by inhibition of the electrogenic flux whereas the decline of the contribution seen at higher K-concentrations is due to a decrease of the membrane resistance (increment of potassium permeability) rather than to a fall of the electrogenic flux

It has been shown in a variety of preparations that the Na K pump may be electrogenic and thus contribute directly to cell membrane potentials (Kerkut and Thomas 1965 Koike Brown and Hagiwara 1971 Kuno Miyahara and Weakly 1970 Nakajima and Takahashi 1966 Rang and Ritchie 1968) These studies have been carried out under non steady state conditions in which the electrogenic contribution to the membrane potential was *increased above resting level* by stimulation of the pump activity

It has been a question of debate whether or not the activity of an unstimulated Na K pump could contribute to the transmembrane potential Moreton (19[illegible]) finds no indication of an electrogenic component in the resting neurons of *Helix aspersa* and Thomas (1969) sets an upper limit to the direct pump contribution of

−2 mV in single cells of the same snail. In contrast to these findings the observations of Gorman and Marmor (1970) on the marine snail *Anisodoris nobilis* show direct evidence for an electrogenic addition to the resting membrane potential. Furthermore this contribution was found to be dependent upon the external potassium concentration. Carpenter (1970) gave indirect evidence of a contribution from the resting pump in *Aplysia* neurons. The seemingly greater electrogenic contribution from the Na K pump in marine snails as compared to terrestrial snails may be caused by a higher electrogenic flux, a larger specific membrane resistance or a combination of both.

In this paper results will be presented that are in accordance with the observations from *Anisodoris nobilis*. A potassium dependent electrogenic pump-potential has been found in an identified resting neuron of the snail *Helix pomatia*. This pump-potential has a maximum value of −7 mV at an extracellular potassium concentration of 4 mM. A decline of the pump contribution seen at lower external potassium concentrations is found to be caused by a decrease of the electrogenic flux due to reciprocal inhibition of the pump activity. At higher potassium concentrations an observed fall of the contribution can be ascribed quantitatively to a decreasing specific membrane resistance rather than to a decline of the electrogenic flux which remains constant at a level near —10 peq cm^{-2} sec^{-1} in the range of 6 mM to 12 mM potassium. Agreement is found between the electrogenic flux calculated from modified constant field equations and the flux calculated from Ohm's law. Finally an estimate of the Na K coupling ratio is given.

Calculations During steady state

$$-J_{K\,passive} = J_{K\,active} \quad (1)$$

and

$$J_{Na\,passive} = -J_{Na\,active} \quad (2)$$

β is defined as the coupling ratio of the Na K pump

$$\beta = -J_{Na\,active}/J_{K\,active} = -J_{Na\,passive}/J_{K\,passive} \quad (3)$$

From (3) it follows that

$$J_{Na\,passive} + \beta J_{K\,passive} = 0 \quad (4)$$

Mullins and Noda (1963) inserted equations for the passive net fluxes in eqn (4) and arrived at the following description of the transmembrane potential in the presence of an electrogenic pump

$$V = \frac{RT}{F} \ln \frac{\beta P_K[K]_o + P_{Na}[Na]_o}{\beta P_K[K]_i + P_{Na}[Na]_i} \quad (5)$$

P is the permeability of the ion indicated by the subscript, V is the membrane potential, i and o refer to the inside and the outside of the membrane. F, R and T have their usual meanings.

Subtraction of (1) from (2) gives

$$J_{Na\,passive} + J_{K\,passive} = -(J_{Na\,active} + J_{K\,active}) = -J_{el} \quad (6)$$

J_{el} is the electrogenic flux produced by the Na K pump and is negative when β is greater than one. If we insert the following constant field equation for the net flux of the j th ion into (6)

$$J_j = \frac{P_j([j]_i e^{FV/RT} - [j]_o) FV}{(1 - e^{FV/RT}) RT}$$

we arrive at the following expression

$$V = \frac{RT}{F} \ln \frac{P_K[K] + P_{Na}[Na] - J_{Na}RT/FV}{P_K[K]_i + P_{Na}[Na]_i - J_{Na}RT/FV} \qquad (8)$$

see Moreton (1969) This equation may be reduced if two approximations are made

a P_{Na} is less than P_K and $[Na]_i$ is less than $[K]_i$ Therefore the term P_{Na} $[Na]_i$ is excluded from the denominator

b The denominator is greater than the numerator Consequently the term $J_{Na}RT/FV$ does not contribute as significantly to the former as to the latter and may be omitted from the denominator

Eqn (8) may now be written as

$$e^{FV/RT} = \frac{[K]}{[K]_i} + \frac{P_{Na}[Na]}{P_K[K]_i} - \frac{J_{Na}RT}{FV\,P_K[K]_i} \qquad (9)$$

in which the last term will be referred to as the sodium pump term as suggested by Moreton (1969) Note that if β is greater than one and J_{Na} has a negative value the presence of the pump term represents a hyperpolarization If β is equal to one or if the Na K pump is inhibited by ouabain (Schatzman 1953) and J_{Na} thereby eliminated eqn (9) reduces to

$$e^{F(V - \Delta V)/RT} = \frac{[K]}{[K]_i} + \frac{P_{Na}[Na]}{P_K[K]_i} \qquad (10)$$

$(V - \Delta V)$ is the membrane potential measured in a ouabain containing Ringer's solution ΔV is the electrogenic contribution to the membrane potential and is related to the depolarization which occurs as a result of application of ouabain

In the development so far chloride ions have been completely neglected This is justified by the observation of Kerkut and Meech (1965) that the ratio of the chloride concentrations on the two sides of the membrane is of little importance to the magnitude of the transmembrane potential. Furthermore we can justify the above two approximations as well as neglecting chloride by plotting $e^{F(V - \Delta V)/RT}$ as a function of [K] Provided that the P_{Na}/P_K ratio and the internal potassium concentration are constant throughout the change in [K] such a plot should reveal a straight line from which P_{Na}/P_K and $[K]_i$ can be derived This is indeed the case for *H aspersa* as shown by Moreton (1969) and also applies to *H pomatia* as will be seen later (Fig 4 b)

Subtraction of (10) from (9) gives

$$(e^{FV/RT})_{pump} - (e^{F(V - \Delta V)/RT})_{no\ pump} = \frac{-J_{Na}RT}{FV\,P_K[K]_i} \qquad (11)$$

The electrogenic flux may be calculated from this eqn the left hand side can be evaluated from measurements of membrane potential made in normal and in ouabain containing Ringer's solutions $[K]_i$ may be determined from the plot described by (10) and P_K can be computed from the following expression

$$\left(\frac{dI}{dV}\right)_{I \to 0} \quad G = \frac{F\,P_K(-V)w\,y}{R\,T\,(w - y)} \qquad (12)$$

where $w = [K] + P_{Na}/P_K[Na] + P_{Cl}/P_K[Cl]_i$

and $y = [K]_i + P_{Na}/P_K[Na]_i + P_{Cl}/P_K[Cl]$

(Hodgkin and Katz 1949) G is the total specific membrane conductance

If we use approximation a and b once more assume that chloride ions do not contribute significantly to G w and y can be reduced

$$w = [K] + P_{Na}/P_K[Na] \quad y = [K]_i$$

P_{Na}/P_K and $[K]_i$ are found from (10) G may be determined by measurements of the total membrane resistance and P_K can be calculated and inserted in (11) to give J_{Na}

Alternatively J_{Na} may be derived directly from Ohm's law (13)

$$\Delta V = R_M\,F\,J_{Na}$$

R_M is the specific membrane resistance

The computation of J_{Na} from (11) is based on measurements of V, $V - \Delta V$, total membrane resistance and cell surface area It is connected to the approximations a and b and the assumption of no chloride influence plus the constant field assumption J_{Na} derived from Ohm's law is based

on the 5 parameters only Therefore if the 2 values of J_1 are equal it is an indication that the assumptions have not seriously influenced the results provided that it is justifiable to apply Ohm s law to the system Rapoport (1970) showed that Ohm s law is an oversimplification in the present situation and should be corrected by a term involving the change of diffusional potential which is associated with the voltage dependent conductance changes that occur with the electrogenic hyperpolarization

$$\Delta V = \left(\frac{\Sigma g_j E_j}{\Sigma g_j} - V_{diff}\right) + \frac{F J_1}{\Sigma g_j} \qquad (14)$$

V_{diff} is the diffusion potential determined by the Goldman equation E_j is the equilibrium potential for the jth ion and g_j is the corresponding conductance The error introduced by neglecting the conductance term in the parenthesis will be estimated in the discussion and the validity of (12) in the presence of an electrogenic flux will be considered

Methods

Identification of the giant neurons used for electrical measurements The greater part of this work was carried out on a cell which appears to be one of the largest in the *Helix pomatia* subesophageal ganglia It is a giant cell situated anteriorly in the left parietal ganglion next to the thin septum of connective tissue separating the left from the right parietal ganglia It will be referred to as cell I For comparison some of the measurements have been repeated on a spontaneously firing cell lying in the right parietal ganglion near the common entrance of the right internal and external pallial nerves (Schmaltz 1914 Kunze 1971) This cell (which will be called cell II) was found to differ from cell I in several aspects The preparation technique used was essentially the same as that described by Kerkut and Thomas (1965)

Experimental chamber and bathing solutions The preparation was pinned down in a perspex chamber closely resembling the one described by Moreton (1968) in which the bathing solutions could be changed The Ringer s solutions were prepared as described by Kerkut and Thomas (1965) Solutions were made with potassium concentrations of 0 2 4 6 8 and 12 mM In parallel to these six solutions another set was made up in which 10^{-4} M ouabain had been added (Merck catalogue no 7896) A stock solution of 10^{-2} M ouabain in Ringer with zero potassium was kept in the refrigerator and diluted 100 times in the respective solutions just before use

Potential measurements Intracellular potential measurements were made with glass microelectrodes having tip diameters of about 0 2 µm Electrode resistance was measured by differentiation of a triangular voltage impulse (Lettvin *et al* 1958) and selected within an impedance range of 5 to 20 MΩ Electrodes were filled with 2 5 M KCl using a destillation filling technique (Zeuthen 1971) Three microelectrodes were employed in each experiment Two electrodes were inserted in the same cell (I or II) One was used for measurement of membrane potential the other for intracellular current injection The third electrode was placed inside the other cell (II or I) for recording its membrane potential The two potential recording electrodes were each connected to the input of a preamplifier provided with neutralization of input capacitance (Bioelectric Instrument Inc type NF1) The signal was displayed on a Tektronix oscilloscope type 502A Continuous recordings were made on a Watanabe Multi Corder model MC611 and in some cases on a Clevite Brush recorder Mark 220 The overall frequency response of the recording readout was flat (maximum high frequency attenuation of 3 dB) up to 125 Hz when the latter pen recorder was used The action potential recorded on the Watanabe penrecorder were greatly attenuated (Fig 2)

Current injections Current pulses were generated by a current source based on operational feedback and having the following specifications Output impedance greater than $10^{?}$ Ω leakage current less than $10^{-?}$ A frequency range dc to 20 kHz output voltage greater than ±5 V continuously variable current output with in ±100 nA accuracy and linearity 1 % The current source could be connected to an outer commandvoltage generator When constant current injections of duration less than 1 s were used a Multitim (DISA Elektronik A S Herlev Denmark) was used as a commandvoltage generator When continuous current voltage characteristics of the membrane were recorded a Wavetek Function Generator Model 115 was used to give the linearly varying current injections For constant current injections lasting longer than 1 s the current source was operated with a manual switch The injected current was measured by connecting the Ag/AgCl reference electrode to the virtual ground of a current to voltage operational amplifier and displaying the calibrated output on the oscilloscope and/or pen recorder

Measurement of membrane resistance and capacitance The total resistance of the resting cell membrane was estimated by injecting small hyperpolarizing square current impulses (−1 to −2 nA) Injections were made small and hyperpolarizing in order to minimize voltage dependent conductance changes and were made long enough to allow full charging of the membrane When membrane resistance was converted into specific membrane resistance the cell surface was estimated as the area of a sphere having the same diameter as the cell Membrane capa-

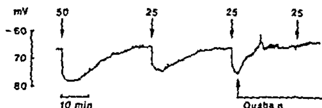


Fig 1 The effect upon the membrane potential of increments of internal sodium concentration in cell I Mechanical injections of Na lasting for about 1 s were carried out at the arrows The numbers indicate the amount of pequiv of Na injected in the cell

measured by injecting a small hyperpolarizing constant current and measuring the initial slope of the voltage change As pointed out by Connor and Stevens (1971 c) all the conductance systems of the membrane are kept constant during this procedure The capacitance is then given by the ratio between the applied current and the initial rate of voltage change

Na load n_a of cells Cell I was loaded with Na by mechanical injection of 1 M NaCl through a glass micropipette broken to a tip diameter of approx 2 μm The NaCl solution was drawn 3 to 4 mm into the tip of the pipette and injected by applying pressure to the shank of the pipette via an air filled polyethylene tube The injected volume was roughly estimated from the displacement of the light reflecting meniscus formed by the solution and from the diameter of the pipette tip Both parameters were measured with an ocular micrometer

Results

Hyperpolarizations following increases of intracellular sodium concentration (non steady state)

The activity of the Na K pump may be enhanced above the resting level by increase of intracellular Na concentration It may be specifically decreased by the binding of certain heart glycosides to the pump sites of the membrane (Schatzmann 1953 Elliot and Keynes 1969) The internal Na concentration was increased in two different ways in the two investigated cells In cell I the concentration rise was produced by the mechanical injection of NaCl and in cell II by repetitive action potentials

Cell I The effect of injections of approximately 50 and 25 peq Na is shown in Fig 1 Marked hyperpolarizations of −12 and −8 mV follow the injections The time constant of the repolarizing phase (τ = 11 min) is considerably larger than the one measured by Thomas (1969) after electrophoretic sodium injections in a giant cell of the closely related *H aspersa* (τ = 4 4 min) Fig 1 also shows that the application of 10^{-4} M ouabain shortly after the sodium injection causes a block of the hyperpolarization Subsequent injection of Na had no hyperpolarizing effect

Cell II This cell showed a regular firing pattern as seen in Fig 2 The small hyperpolarizations following every burst of activity could be substantially enhanced by increasing the firing rate with continued depolarizations of the membrane This is shown in Fig 2 where currents of 2 4 and 6 nA were passed through the second intracellular electrode The tetanus resulting from each depolarization is followed by a hyperpolarization of −10 −13 and −15 mV respectively Ouabain reduced the post tetanic hyperpolarizations to a small size and replaced the regular firing pattern of Fig 2 b by irregular alternations of action potentials and subliminal depolarizations Corresponding posttetanic hyperpolarizations have been observed in mammalian nerve fibres Den Hertog Greengard and Ritchie 1969 Kuno et al 1970) and in crayfish stretch receptor neurons (Nakajima and Takahashi 1966 Sokolove and Cooke 1971) and have been ascribed to the activity of an electrogenic

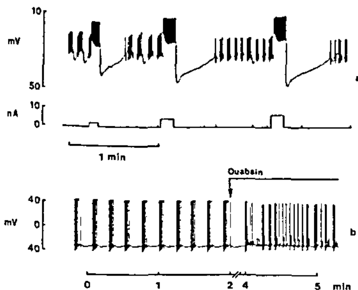


Fig 2 Effect of repetitive firing on the membrane potential of cell II The cell had a spontaneous firing pattern as seen in the left part of 2 b which is the tracing from the Brush recorder The action potentials from the same cell in the pen recording of 2 a are reduced to a few mV The cell is stimulated by injections of constant current impulses the strength and duration of which are shown in the middle tracing Marked hyperpolarizations follow the periods of increased firing rate The right hand part of 2 b shows that the regular firing pattern was interrupted when ouabain was added to the solution bathing the cell

Na K pump stimulated by the Na loading effect of the action potentials Similarly the hyperpolarizations following mechanical Na injection can be explained as the effect of a Na K pump working with a Na K ratio greater than one Alternative interpretations will be mentioned later

Dependency of the membrane potential upon the outer potassium concentration

As Ringer s solutions containing different K concentrations were perfused through the experimental chamber the membrane potential of cell I changed in a manner shown in Fig 3 After each value of $[K]_o$ 4 mM Ringer was introduced into the chamber This allowed for small corrections of V values as the 4 mM levels varied slightly during the experiment (within 5 mV) No $[K]_o$ values higher than 12 mM were used in order to ensure the full reversibility of V with return to 4 mM $[K]_o$

After having made measurements in normal Ringer s solutions with different $[K]_o$ values the same sequence was repeated with 10⁻ M ouabain added to the media This gave a new set of V values at a depolarized level The depolarization commenced within one minute after the application of ouabain and terminated within 10 min The new level of V remained constant (for $[K]_o$ constant=4 mM) throughout the entire period during which the subsequent $[K]_o$ changes were made The rate of potential change following perfusion with a new Ringer s solution rather small compared to a similar measurements made by others (Moreton

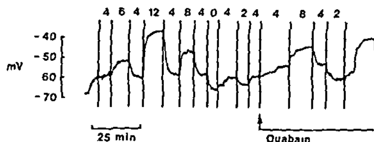


Fig 3 Pen recording of the membrane potential of cell I during variation of external potassium concentration Concentrations are indicated in mM by the numbers at the top of the figure A depolarization is seen at the application of ouabain

1969 Gorman and Marmor 1970) This was due to a very slow exchange of solutions in the chamber necessary to minimize disturbances to the electrodes and the cell In most cases cell I did not show any spontaneous activity even at the highest K concentrations although all experiments were carried out at room temperature

The potassium dependency of the membrane potential of cell I is shown in Fig 4 a The curves show the mean values of 35 experiments obtained in normal and ouabain containing Ringer s solutions $e^{FV/RT}$ vs $[K]_o$ is plotted in Fig 4 b It is seen that the values for ouabain treated cells define a straight line as predicted by eqn (10) This indicates that the simplifications used to obtain eqn (10) have not seriously influenced its validity It further means that P_{Na}/P_K and $[K]_i$ are constant throughout the experiment and may be calculated from the slope and the x or y axes intercept of the line The two parameters were calculated for each experiment after fitting the best line to the experimental results via a least square regression analysis The mean of all such calculations are summarized in Table I where the values for V $[K]_i$ and P_{Na}/P_K from cell I and II of *H pomatia* are compared to those obtained from *H aspersa* (Moreton 1968) It is noteworthy that whereas $[K]_i$ is roughly equal in cell I and II the membrane potential is significantly less negative in the latter and the calculated values of P_{Na}/P_K accordingly larger (about twice as big)

The steady state contribution from the electrogenic Na K pump to the membrane potential

The vertical distance between the two curves in Fig 4 a is a direct expression of the depolarizing effect of ouabain upon the membrane potential Since it was found in Fig 1 and 2 that the Na K pump can contribute to the membrane potential this difference (ΔV) is assumed to be equal to the steady state hyperpolarization of a Na K pump working with an electrogenic outflux of Na (see Discussion) As seen in Fig 4 a this electrogenic pump potential has an increase from zero at $[K]_o = 0$ mM to -7 mV at $[K]_o=4$ mM and then declines towards zero as the K-concentrations increase The deviation from linearity of the lower curve in Fig 4 b is an implicit expression of the electrogenic pump potential This deviation which is described by eqn (11) begins at an outer K-concentration of zero and has a maximum at 6 mM

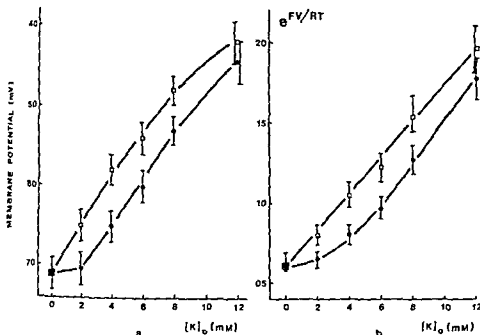


Fig 4 The relationship between the membrane potent al of cell I and the external pota sium concentration Closed circles are values obtained in normal and open squares in ouabain conta ning Ring r s solut ons a Each po nt represents the mean of 35 expts with S E of nean shown b $e^{FV/RT}$ is calculated as a function of [K] from the values in a The straight line has been fitted by a least square regression analysis from which $[K]_i$ and P_N/P_K may b found (see text Lower curve shows a deviation from l nearity wh ch has a maximum at 4 6 mM [K] and decreases rap dly with falling and slowly with rising K concentrations

TABLE I Comparison betw en m mbrane potent al nternal potas um con entration and Na K permeab lity ratio n neurons of *H asp rsa* and tw o ident fi d cells of *H p mat*

	H pomat a		H aspersa
	C ll I	C ll II	
Membrane potent al (mV)	−65 0 ± 1 5 * N = 41	−47 5 ± ? ? N = 5	40 8 ± 1 1 = 7
Intern l potass um concn (mM)	93 ± 7 N = 35	113 ± 10 N = 5	9? 9 ± 4 3 N = 7
P /P_K	0 067 ± 0 007 N = 35	0 17 ± 0 0? N = 5	0 18 ± 0 015 N = 57

M reton 1968
F gures are g en w th S E of mean N = number of experiment [K] = 4 mM

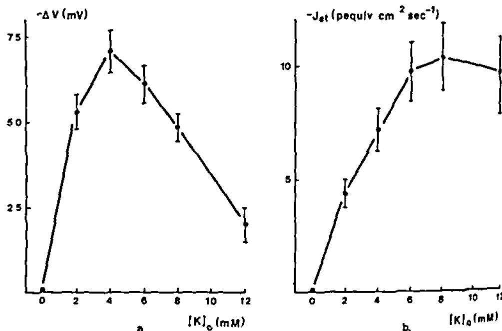


Fig 5 The electrogenic contribution to the membrane potential and the corresponding active net flux of the Na K pump both as a function of external potassium concentration a The difference at each level of [K] between the membrane potential measured in normal and in ouabain containing Ringer's solutions was found in each experiment Each point represents the mean of all such differences ±S E of mean b The electrogenic flux has been calculated by use of Ohm's law from the values of ΔV in Fig 4 a and of R_M in Fig 7 a

$[K]_o$ after which there is a small convergence of the linear and non linear curves These results differ from those of Moreton (1969) who found that the sodium pump term of (9) can be regarded as a constant when J_{el} is constant Furthermore he did not observe any decline of ΔV above 3 mM $[K]_o$ in Na loaded cells The findings described above do however fit with the observations of Gorman and Marmor (1970) who found a maximum deviation from linearity in the marine snail *Anisodoris nobilis* at around 25 mM $[K]_o$ and a clear convergence of the curves for increasing as well as decreasing $[K]_o$ values In order to interpret the potassium dependency of ΔV in terms of an electrogenic flux according to eqns (11) and (13) it is necessary to know the membrane resistance in the potential range in which V varies during the $[K]_o$ changes

The membrane resistance

Fig 6 shows a continuous current voltage relationship for cell I The potential function used to command the current injection is seen in the inset The absence of action potentials in the first quadrant of the plane where the cell is depolarized does not imply that the membrane was inexcitable It could be excited by injecting square wave depolarizing current pulses Fig 6 however was obtained at a frequency low enough (0 1 Hz) to cause accomodation of the membrane Because of the low frequency rapid inward Na and Ca-currents do not influence the shape of the curve

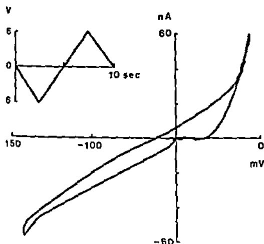


Fig 6 Continuous pen record ng of the current voltage relationship of cell I Membrane potential is shown on the abcisse and injected current in the ordinate The potential function monitoring the current injection is shown in the inset Origin taken as resting membrane potential and zero appl ed current

(Geduldig and Gruener 1970) It has been shown, however that one of the two K conductance systems present in snail neurons requre several seconds to settle at a fully inactivated steady level (Connor and Stevens 1971 a b c, see also Neher 1971) Therefore each point of the graph in Fig 6 wich is governed by the K conductance systems does not express a stationary level Consequently the curve demonstrates a hysteresis effect

Conversion of Fig 6 into membrane resistance as a function of membrane potential shows that the maximum dR_M/dV occurs in the region in which the potential varies during the $[K]_o$ changes *i e* near the resting membrane potential Thus the membrane resistance had to be measured at each level of $[K]_o$ The results of such measurements are shown in Fig 7 a in which the specific membrane resistance is shown versus $[K]_o$ As seen R_M is considerably higher than the corresponding value for the squid giant axon 1 kOhm cm^2 (Cole and Hodgkin 1939) but not as high as the 100 kOhm cm^2 reported from neurons of *Aplysia californica* (Carpenter 1970)

The sodium and potassium permeability of the cell

The measurements of resting membrane resistance shown in Fig 7 a may be used to calculate the potassium permeability of the cell This has been done by converting the membrane resistance into specific membrane conductance and inserting this in eqn (12) The P_{Na}/P_K and $[K]_i$ values used in the equation were the averages taken from Table I and the V values corresponding to each level of $[K]_o$ were the mean of all experiments (Fig 4 a) The results of the calculations for cell I are shown in Fig 7 b P_K has a minimum of $9\ 5\times10^{-7}$ cm s^{-1} at $[K]_o=2$ mM With the Na K permeability ratio from Table I this corresponds to a P_{Na} of $0\ 64\times10^{-7}$ cm s^{-1} It may be of interest to note that in smooth muscle cells in taenia coli from the Guinea Pig $P_K=11\times10^{-}$ cm s^{-1} and $P_{Na}=1\ 8\times10^{-8}$ cm s^{-1} (Casteels 1969) while in the frog skeletal muscle $P_K=1\ 4\times10^{-6}$ cm s^{-1} (Hodgkin and Horowitz 19

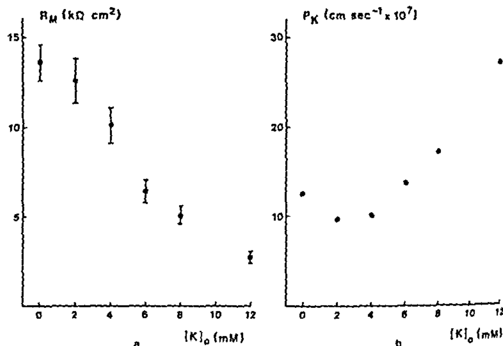


Fig 7 Dependency of the resting membrane resistance (a) and of the potassium permeability (b) upon external potassium concentration in cell I R_M is shown with S E of mean P_K has been calculated on the basis of R_M see text

The electrogenic flux in the resting neuron

The relation between ΔV and J_{et} may be described by Ohm's law Thus when the values of the electrogenic pump potential (Fig 5 a) and of the specific membrane resistance Fig 7 a) are known one can immediately calculate the corresponding values of the electrogenic flux at each level of $[K]_o$ The results are shown in Fig 5 b The flux reaches a maximum near -10 peq cm^{-2} s^{-1} at $[K]_o = 6$ mM and s ays at this level with increasing potassium concentrations Below 6 mM there appears to be a linear inhibition of the electrogenic flux which reaches zero at $[K]_o = 0$ mM Based on a similar calculation Carpenter (1970) suggests a steady state electrogenic flux in Aplysia of 1 peq cm^{-2} s^{-1} and based on flux measurements Casteels Droogmans and Hendrickx 1971 b find a value of -9 peq cm^{-2} s^{-1} for the same parameter in smooth muscle of the guinea Pig taenia coli

An alternative way of computing J_{et} is by the use of eqn (11) The left hand side of this equation is found from Fig 4 b and P_K is given in Fig 7 b The results of such calculations are shown in Table II along with the J_{et} values derived from Ohm's law It appears that there is a general agreement between the fluxes derived from the modified constant field equations and those from Ohm's law

The individual passive Na and K fluxes can be calculated from eqn (7) if the membrane potential the ion permeabilities and the intra and extracellular concentrations are known In this case $[Na]_i$ is unknown An intracellular Na activity

TABLE II Relationship between the electrogenic flux derived from Ohm s law and from constant field equations and the external potassium concentration

external potassium concn (mM)	electrogenic flux Ω (pequiv cm⁻ s)	electrogenic flux* (pequiv cm⁻ s⁻)	$J'_{K\ pass}$ (pequiv $cm^{-2} s^{-1}$)	$J_{K\ pass}$ (pequiv $cm^{-2} s^{-1}$)	$J'_{Na\ pass}$ * (pequiv cm⁻ s⁻)	$J_{Na\ pass}$ (pequiv cm⁻ s^{-1})	electrogenic flux* (pequiv cm⁻ s⁻)
0	0	0	−20 2	−20 2	19 7	19 7	0
2	−4 4	−3 7	−11 4	−14 4	15 1	14 2	−3 9
4	−7 2	−5 6	−9 4	−14 [illegible]	14 9	13 6	−6 1
6	−9 7	−7 7	−10 3	−17 5	18 7	17 3	−8 6
8	−10 4	−8 5	−14 7	−2[illegible] 9	20 5	19 2	−9 5
12	−9 6	−8 1	−24 8	−33 4	29 6	28 5	−9 7

Ω calculated from Ohm s law
* — — eqn (11)
** — — — (15)

J′ is the flux when the pump is active
J — — — — — inhibited

of 13 mM has been found by Sorokina (1966) with the use of Na selective micro electrodes while a value of 3 5 mM has been determined by Thomas personal communication For the present calculation Thomas value of 3 5 mM is used The P_{Na} and P_K values are taken from Fig 7 b and the permeability ratio from Table I The P_K values were calculated for the membrane potential corresponding to an active pump situation but are assumed to be the same when the pump is inhibited The results of calculations of the passive Na and K fluxes in the situation where the pump is inhibited by ouabain are shown in Table II (J_K and J_{Na}) The values show that J_K+J_{Na} at each level of $[K]_o$ are not equal to zero but to some small constant (ε) This might be due to the errors introduced by assuming $[Na]_i=3\ 5$ mM and equal permeabilities with and without pump an experimental error or a small leakage flux (possibly Cl) In the case of an active pump J_K and J_{Na} acquire new values according to the excess electropotential gradient caused by ΔV and become J'_K and J'_{Na} which are calculated in Table II According to (6) we can now write that

$$\text{Without pump } J_K+J_{Na}=\varepsilon$$
$$\text{With pump } J'_K+J'_{Na}=-J_{el}+\varepsilon$$

Subtraction of these expressions give

$$(J'_K-J_K)+(J'_{Na}-J_{Na})=-J_{el} \qquad (15)$$

In this way a third set of J_{el} values has been calculated Table II shows that there is agreement between this and the two other ways of estimating J_{el}

Discussion

Throughout this paper the effects of sodium injection and of ouabain addition have been ascribed to the activity of an electrogenic Na K pump working with a Na K ratio greater than one The results however might find alternative explanations and a verification of the above conclusion is necessary and will be attempted h

1:1 coupled Na,K pump. The depolarization of the membrane potential seen in Fig. 3 after application of ouabain might be explained as a beginning equalization of the ion concentrations across the membrane when the Na,K pump (electroneutral) is no longer present to maintain the concentration gradients. This would diminish the negativity of the membrane potential. If this were the correct explanation the depolarization would begin immediately after ouabain addition and would continue at a high rate until all differences in concentration were eliminated to a degree expected from a Donnan distribution. The membrane potential would accordingly decrease rapidly to a very small value. The depolarization did indeed commence within 1 min after the application of ouabain, but the new level of membrane potential, reached within ten minutes, persisted within a few mV for more than two hours.

The hyperpolarizations shown in Fig. 1 and 2 following the sodium injections could result from an increase in $[K]_i$ caused by the stimulated activity of a 1:1 coupled Na-K pump. This however is not likely. A hyperpolarization of −12 mV such as the one in Fig. 1 would, calculated on the basis of eqn. (10), require an immediate increase in internal potassium concentration of 60 % of the original level (93 mM), which is not likely to be the case after an injection of only 50 peq Na.

Permeability changes. The effect of ouabain shown in Fig. 3 might be ascribed to an immediately occurring permeability change caused by the drug. This is contradicted by the findings of Gorman and Marmor (1970) that at low temperatures (where the pump is already inhibited) no depolarizing effect could be observed as well as by the observation of Casteels *et al.* (1971 a). The deviation from linearity at low potassium concentrations of the curve obtained in normal Ringer (Fig. 4 b) could also be explained by a permeability change, e.g. a fall in P_K. This would tend to depolarize the membrane potential further from the K equilibrium potential (−79 mV at $[K]_o$ = 4 mM) and give the observed deviation. If this were an adequate explanation it would be expected that the ouabain curve in Fig. 4 b should show a similar deviation from linearity. This is not supported by the experimental results. Furthermore Fig. 7 b shows that P_K has a small (though insignificant) tendency to rise at [K] = 0 mM.

An electrogenic Na/K pump. The considerations mentioned above all seem to favour the concept of an electrogenic Na-K pump as the most likely cause of the presented observations. Since the effect of the pump was always hyperpolarizing, the Na/K ratio must be greater than one and is estimated below in three different ways.

According to (3) the Na/K ratio of the pump can be expressed as $-J_{Na}/J_K$ (Table II). The average of all ratios at each level of $[K]_o$ is 1.46 or nearly 3:2. Another way of calculating the ratio can be applied to the electrogenic hyperpolarizations of Fig. 2. The electrogenic charge Q_{el} carried out of the cell during the post-tetanic hyperpolarization as uncompensated sodium ions can be found as the area under the hyperpolarization divided by the membrane resistance

$$Q_{el} = Q_{Na\,active}(1 - 1/\beta) = \int V\,dt/R_M \qquad (11)$$

$Q_{Na\,active}$ is the total amount of excess Na charge carried actively out of the cell

Part of ΔV is accounted for by the change in diffusion potential that follows the alterations of membrane conductance when the cell is hyperpolarized by the pump This change is described by the conductance term in parenthesis of eqn. (14) the magnitude of which will be estimated here

If we insert $I_j = g_j\,(E_j - V)$

and $\Sigma g = g_K + g_{Na} + g_{Cl}$ in the conductance term of eqn (14) it can be written as

$$\frac{E_K I_K/(E_K-V) + E_{Na} I_{Na}/(E_{Na}-V) + E_{Cl} I_{Cl}/(E_{Cl}-V)}{I_K/(E_K-V) + I_{Na}/(E_{Na}-V) + I_{Cl}/(E_{Cl}-V)} = V_{diff}$$

Since it was found from the results that I_{Cl} was negligeable the Cl terms may be omitted When eqn (3) is inserted the term reduces to

$$\frac{[E_K\,(E_{Na}-V)]/[-\beta\,(E_K-V)] + E_{Na}}{(E_{Na}-V)/[-\beta\,(E_K-V)] + 1} = V_{diff}$$

E_K can be found since $[K]_i$ is known E_{Na} is 79 mV if $[Na]_i$ is assumed to be 3 5 mM When these values are inserted along with $\beta = 3/2$ it turns out that the correction of the electrogenic contribution to the membrane potential due to conductance changes is negligeable This should be compared to the rather modest pump potential of -7 mV In smooth muscle cells (Casteels *et al* 1971 b) and in some mammalian neurons (Carpenter 1970 Gorman and Marmor 1970) where electrogenic contributions in the order of -20 mV are reported one would expect a large change in diffusion potential associated with the electrogenic hyperpolarization These large steady state contributions are undoubtedly connected to the higher specific membrane resistances of the cells as compared to the *H pomatia* neurons It seems unlikely that one should be able to detect any effect on the membrane potential from the Na K pump in cells with lower membrane resistances (such as the squid giant axon unless the pump works at a very high rate

Eqn 12 which has been used in the calculation of P_K from the total membrane resistance is derived under the assumption of zero net passive ion flux (Hodgkin and Katz 1949 Although it should not in theory be applied to a situation in which an electrogenic flux is present the resulting error in P_K values obtained from eq (12) seem to be small in the present case since it turns out that I_K does not change much as a result of the electrogenic hyperpolarization This is evident from the fact that the diffusional potential is almost constant during the electrogenic hyperpolarization and from the observation that the resting membrane resistance also remained practically constant when the pump was inhibited In addition it was found that the behaviour of the sodium pump term at varying potassium concentrations could largely be accounted for by the calculated dependency of I_K upon the external potassium concentration This was indicated by the agreement between the electrogenic fluxes computed directly from Ohm's law and from the modified constant field equations that included the sodium pump term in which I_K was inserted It therefore seems that the simplified eqn 9, with reasonable accuracy can be used to calculate the electrogenic flux

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Cerebral Energy State and Cerebral Venous P_{O_2} in Experimental Hypotension Caused by Bleeding

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Abstract

Eklof B V MacMillan and B K Siesjo *Cerebral energy state and cerebral venous P_{O_2} in experimental hypotension caused by bleeding* Acta physiol scand 1972 *86* 515—527

Arterial hypotension was induced by bleeding lightly anesthetized rats to mean arterial blood pressures of 60 and 45 mm Hg respectively with subsequent measurements of arterial and cerebral venous gas tensions of extra and intracellular acid base parameters as well as of parameters necessary for an evaluation of the energy state of the brain tissue In none of the groups were there significant changes in extra or intracellular pH indicating that a non chemical mechanism was responsible for the autoregulation of flow Most of the animals in the 60 mm Hg group and about half of those with a blood pressure of 45 mm Hg showed a normal metabolic pattern in the brain In the remaining animals there were moderate increases in lactate and simultaneously slight decreases in the charge potential of the adenine nucleotides of the tissue due to moderate increases in ADP and AMP Such changes occurred at a venous P_O of about 25 mm Hg Since other results indicate that the cerebral energy state fails at higher mean arterial blood pressures and at higher P_{vO} values provided that the tissue is acidotic it is concluded that the efficient energy homeostasis observed in the present experiments may have been due to the fact that no tissue acidosis developed

When the mean arterial blood pressure is accidentally decreased in patients or deliberately lowered in experimental animals the cerebral blood flow (CBF) is upheld close to normal values until the blood pressure falls below 50—70 mm Hg (Lassen 1959 Rapela and Green 1964 Haggendal and Johansson 1965 Harper 1965) When the perfusion pressure is decreased further the CBF falls but major biochemical signs suggestive of a derangement of the cerebral energy metabolism do not occur until the blood pressure falls below about 40 mm Hg (Siesjo and Zwetnow 1970 a Siesjo *et al* 1971) In some animals a slight increase occurs in the tissue lactate content and in the lactate/pyruvate ratio even at perfusion pressures exceeding 40—50 mm Hg but this lactate accumulation does not seem to induce a

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significant fall in the extra- and intracellular pH (Siesjö and Zwetnow 1970 a, see also Siesjo and Zwetnow 1970 b). Thus, since the autoregulation of the cerebral blood flow cannot be related to acidosis, the experiments quoted supported the view of a nonchemical (myogenic) autoregulatory mechanism (see Folkow 1963, Ekström Jodal *et al.* 1970).

Recent experiments from our laboratory have shown that the cerebral energy state fails at relatively high cerebral venous P_{O_2} values when the cerebral perfusion pressure is moderately decreased in animals with bilateral carotid artery ligation (Eklöf and Siesjo 1972) or when the blood pressure is reduced during moderate hypercapnia (Eklöf, MacMillan and Siesjö 1972 a). The experiments have led to the tentative hypothesis that abolished autoregulation, possibly caused by tissue acidosis, predisposes the tissue to regional ischemia, i.e. to a non-homogenous decrease in flow, when the cerebral perfusion pressure falls (see Siesjo, Eklöf and MacMillan 1972).

In the present experiments, rats were bled to mean arterial blood pressures of 60 and 45 mm Hg, respectively, for 30 min, and measurements were made of arterial and cerebral venous gas tensions, of extra- and intracellular acid base parameters as well as of parameters necessary for an evaluation of the energy state of the tissue. The experiments had two main objectives. The first objective was to study if deep arterial hypotension causes tissue acidosis at constant tissue CO_2 tension. The second objective was to evaluate possible changes in the energy state of the tissue, using sensitive fluorometric techniques for measuring the adenine nucleotides in the tissue, and to relate the energy state to the venous oxygen tension.

Methods

The experiments were devised to study P_{O_2}, P_{CO_2} and pH in arterial and cerebral venous blood, lactate, pyruvate, P_{CO_2} and $[HCO_3^-]$ in cisternal CSF, as well as a number of parameters necessary to evaluate the energy state and the acid-base status of the tissue. Since enough CSF could not be obtained in one animal for analyses of both lactate, pyruvate, P_{CO_2} and $[HCO_3^-]$, and since it was considered advisable not to determine labile tissue metabolites in animals used for sampling sagittal sinus blood, two parallel series of experiments were run. In the following we will describe the procedures which were the same for all animals as well as those which were specific for the two groups (denoted by A and B, respectively).

Procedures in common. The experiments were performed on male Wistar rats (300–400 g) which were allowed free access to water and rat pellets until operation. Anesthesia was induced with diethyl ether in a closed jar. When the animals were unresponsive to external stimuli they were quickly tracheotomized, injected i.p. with tubocurarine chloride (0.6 mg/100 g) and connected to Starling type respirators. Throughout the experiments, anesthesia was maintained with 0.6 % halothane and the oxygen concentration of the inspired air was kept at 30 %. One femoral artery was cannulated for blood pressure recording with an electromanometer, and the other femoral artery was cannulated for sampling of arterial blood as well as for bleeding the animals into a constant pressure reservoir. A mercury thermometer was inserted into the rectum for measurements of the body temperature, which was kept at 37.0 °C by means of intermittent heating. A skin incision was placed over the cranial vault to accomodate a plastic funnel for later freezing of the brain *in situ* (see Siesjö and Nilsson 1971), and the atlanto-occipital membrane was exposed for later sampling of cisternal CSF. In order to avoid cooling of the brain, both incisions were closed until the sampling of CSF and the freezing of the tissue.

After the operation the animals were allowed a steady state period of about 30 min. During that time at least two sets of arterial blood samples were taken for analyses of pH, P_{CO_2} and P_{O_2}, and the ventilation was adjusted to give a P_{CO_2} of close to 40 mm Hg. When a [illegible]

steady state was present as judged from a P_{CO_2} constancy of within 10 % between two consecutive samples taken at least 10 min apart, the animals were bled into the reservoir which allowed the pressure to be maintained at 60 and 45 mm Hg respectively At 10 20 and 30 min, respectively after the desired blood pressure level was obtained arterial blood was sampled for measurements of pH P_{CO_2} and P_{O_2} at 30 min blood was also collected for subsequent measurements of hemoglobin lactate pyruvate and glucose A control group was run without arterial bleeding and the blood pressure was allowed to attain its spontaneous value In the hypotensive groups the arterial P_{CO_2} decreased even if the ventilation was kept constant In order to avoid too marked a fall in Pa_{CO_2} in the groups kept at a blood pressure of 45 mm Hg the ventilatory volume was decreased In some of the hypotensive animals a more marked fall in Pa_{CO_2} occurred and in order to assure that the animals were comparable these were excluded from the material

Group A In this group a burr hole was placed over the superior sagittal sinus and blood was sampled by puncturing the wall of the exposed sinus with the sharp tip of a glass capillary Blood from the femoral artery and from the sinus was sampled at 0 10 20 and 30 min, respectively for measurements of pH P_{CO_2} and P_{O_2} and at 30 min a CSF sample was withdrawn from the cisterna magna for measurement of P_{CO_2}

Group B In this group the sinus was not exposed the CSF was sampled at 30 min for determination of the total CO_2 content and for the lactate pyruvate and glucose concentrations and the tissue was then frozen *in situ* for subsequent measurements of total CO_2 phosphocreatine ATP ADP AMP glucose G 6-P lactate pyruvate α ketoglutarate malate and glutamate The total CO_2 content was measured on one brain hemisphere and all the other metabolites on the other

Analytical techniques Arterial and venous P_{O_2} P_{CO_2} and pH were measured using microelectrodes operated at 37 0 C with appropriate corrections for temperature differences between the animal and the electrode The pH values were corrected for the suspension effect by adding 0 01 to the values measured (Severinghaus 1965) The CSF CO_2 tension was measured with the micro CO_2 electrode and the CSF CO_2 content with a microdiffusion technique The total CO_2 content of the supratentorial parts of the brain was measured with a microd ffusion method and the tissue parameters listed abo e were determined with specific enzymatic (fluorometric) techniques For further details of procedures and anal tical techniques reference is made to recent communications from the laboratory (S esjo Folbergro a and MacMillan 19/2 Folbergrova MacMillan and Siesjo 1972 a and b MacMillan and S esjo 1972 b)

Calculations The plasma HCO_3 concentrations were calculated from the P_{CO_2} and pH values using a pH-corrected pK value and a solubility factor of 0 030 mmol/kg mm Hg (Severinghaus 1965) The mean tissue CO_2 tension (Pt_{CO_2}) was calculated as

$$Pt_{CO_2} = \frac{Pa_{CO_2} + Pv_{CO_2}}{2} + 1 \qquad (1)$$

and compared to the measured CSF CO_2 tension (Pontén and Siesjo 1966) The CSF HCO_3 concentration was calculated by subtracting from the measured total CO_2 content the product Pt_{CO_2} S where S is 0 0318 mmol/kg mm Hg and the CSF pH was calculated us ng a pH corrected pK value (M tchell Herbert and Carman 1965)

Since the total CO_2 content was measured on one hemisphere and the other metabolites on the other and in o der to a oid erroneous conclusions due to nhomogeneities bet een the two halves of the brain the intrac llular pH was derived both from the CO_2 method and from the creatine phosphokin se (CPK) equilibrium Details of these calculations are given in a recent communication (Siesjo Folbergrova and MacMillan 1972) In prin iple with the CO_2 method the ntracellular HCO_3 concentration is calculated by subtracting from the me sured CO_2 content the amount of CO_2 dissol ed and by correcting for the amount of HCO_3 contained in a 3 % blood and a 15 % ext acellular fluid volume the intr icellul r pH i then derived from the HCO_3 concentration and the Pt_{CO_2} (see also Messeter and Sie j 1971) The CPK method is based on the fact that the CPK equilibrium is pH dependent and pro ided the apparent equilibrium constant is calculated from the pH and from the pho pho creatine (PCr) creat ne (Cr) ATP and ADP concentrations measured in the control group the pH in any [illegible] perimental s tuation may be deri ed from the equation

$$[H^{+}] = \frac{[ATP]_o\ [Cr]_o}{[ADP]_o\ [PCr]_o} \cdot K \qquad (2)$$

where the subscript (o) denotes the total concentrat on of all ionic species of a particula metabol te (Kuby and Noltman 1962 Rose 1968)

The energy charge potential of the aden ne nu leotides (ECP) w s calcul ted ac ord ng the equat on

TABLE I. Mean arterial blood pressure, rectal temperature, blood hemoglobin concentration, arterial pH, P_{CO_2} and P_{O_2} in the two experimental series. In the "A" groups arterial and cerebral venous blood was sampled for measurements of pH, P_{CO_2}, P_{O_2} and CSF was collected for measurements of P_{CO_2}. In the "B" series the superior sagittal sinus was not exposed, and CSF and tissue were sampled for measurements of metabolites (see text). Means ± S.E.

Group	MABP mm Hg	Temp C	Hb g/100 ml	pH	Pa_{CO_2} mm Hg	Pa_{O_2} mm Hg
A	132	37.1	15.5	7.41	39.3	138
n = 8	±3	±0.1	±0.3	±0.01	±0.9	±9
B	125	37.2	16.9	7.39	39.4	143
n = 9	±2	±0.1	±0.4	±0.03	±0.6	±5
A	60	37.1	14.6	7.35	37.8	154
n = 6		±0.1	±0.4	±0.03	±1.[illegible]	±9
B	60	37.1	14.9	7.29	39.3	149
n = 6		±0.1	±0.8	±0.0[illegible]	±1.0	±8
A	45	37.1	14.0	7.18	34.5	138
n = 6		±0.2	±0.5	±0.05	±1.1	±8
B	45	37.1	13.1	7.23	36.0	151
n = 8		±0.1	±0.5	±0.03	±0.9	±5

$$ECP = \frac{ATP + 0.5\ ADP}{ATP + ADP + AMP} \quad (1$$

(Atkinson 1968). The cytoplasmic NADH/NAD⁺ ratio was calculated from pH_i and from the intracellular lactate and pyruvate concentrations, using a K value of $1.11 \cdot 10^{-11}$ (Williamson, Lund and Krebs 1967). The intracellular lactate and pyruvate concentrations were calculated from the total tissue contents after correction for the amount of lactate and pyruvate in extracellular fluid and blood (see Folbergrová, MacMillan and Siesjö 1972 a).

Statistical analyses. Students T test was performed on the experimental groups compared to the normal group. The following symbols are used: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***.

Results

In the following, the results of one series (A or B) are assumed to be applicable also to the experiments of the other. In order to obtain groups with comparable Pa_{CO_2} values, two rats with Pa_{CO_2}s of 38.8 and 40.6 mm Hg were excluded from group B (45 mm Hg), but the metabolite results obtained in these rats will be mentioned below. Table I shows that the blood pressure, the body temperature, the hemoglobin concentration, as well as the arterial pH and gas tensions measured at 30 min were similar in the groups.

Group A

Table II shows the arterial and cerebral venous values for P_{CO_2}, P_{O_2} and pH measured at 30 min. Lowering of the mean arterial blood pressure to 60 and 45 mm Hg respectively led to a progressive decrease in the cerebral venous P_{O_2} and to an increase in the arteriovenous P_{CO_2} difference which was approximately doubled at 45 mm Hg.

TABLE II P_{CO_2}, P_{O_2} and pH in the femoral artery and the superior sagittal sinus and the corresponding A V differences measured at 30 min in the control group and in the groups bled to 60 and 45 mm Hg respectively Means ± S E

MABP mm Hg	P_{CO_2} mm Hg			P_{O_2} mm Hg			pH		
	Art	Vein	Diff	Art	Vein	Diff	Art	Vein	Diff
132 ±3 0 n=8	39 3 ±0 9	49 7 ±0 9	10 5 ±0 3	138 ±9	40 8 ±0 9	97 ±10	7 405 ±0 005	7 365 ±0 009	0 047 ±0 006
60 n=6	37 8 ±1 2	49 4 ±1 1	11 6* ±0 4	154 ±9	35 6* ±1 8	119 ±9	7 352* ±0 026	7 306 ±0 0?3	0 047 ±0 006
45 n=6	34 5 ±1 4	54 9 ±2 9	20 4*** ±2 1	138 ±8	?8 6 ** ±2 0	109 ±9	7 175*** ±0 05	7 104 * ±0 05	0 077* ±0 01

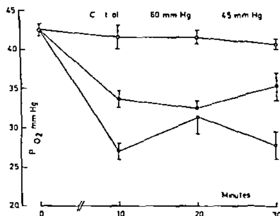


Fig 1 Venous oxygen tensions measured in the superior sagittal sinus before bleeding as well as 10 20 and 30 min after the blood pressure had been reduced to 60 or 45 mm Hg respectively Means ± S E

TABLE III Comparison between the tissue CO_2 tension (P_{tCO_2}) calculated from the arterial and cerebral venous CO_2 tensions and the measured CSF CO_2 tension in the control group and in the hypotensive groups Means ± S E

P_{tCO_2} mm Hg	Control	60 mm Hg	45 mm Hg
Calculated	46 6 ±0 9	44 3 ±1 4	44 7 ±2 0
Measured (CSF)	45 3 ±1 2	44 0 ±1 1	43 3 ±2 4

Fig 1 shows the mean values for cerebral venous P_{O_2} measured at 10 20 and 30 min No systematic changes were observed after the first measurements at 10 min whence it could be concluded that the cerebral venous P_{O_2} s were relatively constant throughout the experimental period However the P_{O_2} varied from animal to animal at any given blood pressure In the 45 mm Hg group the range was between 21 3 and 35 0 mm Hg the first value being the lowest measured in any

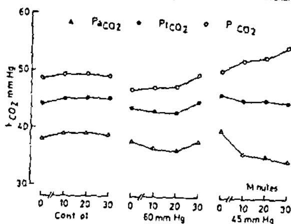


Fig 2 Arterial and venous P_{CO_2} measured in the femoral artery and in the superior sagittal sinus, respectively and calculated tissue CO_2 tension before bleeding as well as 10 20 and 30 min after that the desired blood pressure level had been obtained

The values of Table II allow the calculation of mean tissue CO_2 tensions according to equation (1) Table III compares the Pt_{CO_2} values thus derived and the measured CSF CO_2 tensions There was a good agreement between the two sets of values thus strengthening the assumption that the mean tissue CO_2 tension can be calculated from the arterial and cerebral venous P_{CO_2} values also under hypotensive conditions (*cf* Pontén and Siesjö 1966) Fig 2 shows the arterial and cerebral venous P_{CO_2}s and the calculated mean tissue CO_2 tensions at 10 20 and 30 min in the 3 groups of animals The results indicate that the mean tissue CO_2 tension remained close to normal at all times in the hypotensive conditions

Group B

The P_{CO_2} relations found in group A were used to derive tissue CO_2 tensions in group B Thus in every animal of group B the tissue CO_2 tension was obtained from the measured arterial CO_2 tension by adding the mean difference (Pt_{CO_2} − Pa_{CO_2}) observed in group A (see Fig 1) The derived CO_2 tensions were then used with the measured CSF and tissue CO_2 contents to obtain the CSF and intracellular pH values

TABLE IV D r v d CO ten i ns measured total CO contents and calc il te l val i sf r [HCO] and pH f ternal CSF in control group and in hypot ns e groups M ans ± S E

MABP	P_{CO} mm Hg	[illegible] mm Hg	[HCO] mmol/kg	pH mmol kg	
132 ± 3 n = 9	4[illegible] 7 0[illegible]	28 2 0 3	26 8 0 3	7 31[illegible] 0 006	
60 n = 6	4[illegible] 1 1 0	29 7 0 5	28 2 0 5	7 415 0 008	
45 n = 7	[illegible] 0 [illegible]	29 7 0 2	28 2 ± 0 [illegible]	7 39[illegible] 0 00[illegible]	

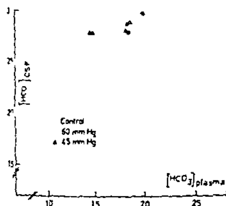


Fig 3

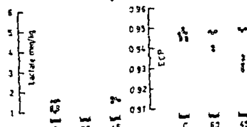


Fig 4

Fig 3 Relation between bicarbonate concentrations (mEqv/kg) in plasma and CSF Individual values for the control group and the group bled to 60 and 45 mm Hg respectively Note constancy of CSF [HCO_3] in spite of decreases in plasma [HCO_3]

Fig 4 Lactate concentrations and energy charge potentials in the brain tissue Individual values for the control group and for the groups bled to 60 and 45 mm Hg respectively Filled circles indicate the two rats excluded from Table VI The dot marked with an arrow denotes 7.37 mEqv/kg

In Table IV are given the derived CSF CO tensions and the measured CO contents together with the calculated [HCO_3] and pH values in CSF There were no significant decrease in [HCO_3] or in pH in any of the hypotensive groups The CSF HCO_3 concentration was upheld in spite of marked decreases in plasma [HCO_3] as shown in Fig 3

Intracellular pH was derived both from the CO data and from the CPK equilibrium (see Methods and Table VI below) When calculating the mean pH values from the CO data (Table V) one markedly low value (6 55) was excluded

TABLE V Derivation of intracellular pH in the brains of control and of hypotensive animals The CO pH was calculated from the tissue CO tension and from the measured CO content (see Methods) The CPK pH was derived from the tissue contents of phosphocreatine ATP and ADP assuming a constant sum for phosphocreatine and creatine of 10 5 mmol/kg (cf Siesjo Folbergrova and MacMillan 1972)

MABP mm Hg	Ptco mm Hg	Tco mmol/kg	[HCO] mmol/kg	pH_i (CO method)	pH (CPK method)
137 ±3 n=9	45 7 ±0 6	13 3 ±0 2	11 8 ±0 3	7 034 ±0 010	
60 n=6	46 1 ±1 0	14 1 ±0 2	13 0 ±0 2	7 073 ±0 009	7 073 ±0 021
45 n=7	48 0 ±0 5	14 4 ±0 3	13 6 ±0 5	7 073 ±0 010	7 010 ±0 030

TABLE VII Lactate and pyruvate concentrations measured in blood and CSF and calculated for the *intracellular* compartment, as well as calculated cytoplasmatic NADH/NAD ratios in control group and in hypotensive groups. Means ± S.E.

MABP mm Hg	Lactate mEqv/kg			Pyruvate mEqv/kg			NADH/NAD 10-
	Blood	CSF	I.c.	Blood	CSF	I.c.	
137 ±3.0 n=9	1.50 ±0.12	2.75 ±0.15	1.40 ±0.11	0.111 ±0.016	0.163 ±0.060	0.105 ±0.008	1.64 ±0.14
60 n=6	6.08 ±1.23	2.89 ±0.09	1.37 ±0.16	0.167 ±0.019	0.165 ±0.004	0.090 ±0.013	2.0 ±0.17
45 n=7	8.34*** ±1.02	3.52 * ±0.12	2.08 ±1.03	0.184 * ±0.010	0.167 ±0.004	0.110 ±0.009	2.24 ±0.92

highly significant increase in the blood lactate content in the hypotensive groups ($p < 0.001$) and in the CSF lactate content in the 45 mm Hg group ($p < 0.01$). There were no significant changes in the CSF pyruvate concentration or in the calculated intracellular lactate and pyruvate concentrations ($p > 0.05$). Although there were small increases in the mean NADH/NAD ratios calculated for the hypotensive groups the changes were not statistically significant. However in those animals in which the lactate content was increased and the ECP value decreased there were also usually NADH/NAD (and lactate/pyruvate) ratios which were increased above the normal range.

Substrate levels. The changes occurring in lactate, pyruvate, ECP and NADH/NAD in some of the hypotensive animals suggested that a critical degree of ischemia was approached. In order to study if there were signs of substrate depletion as well glucose-6-P, α-ketoglutarate, malate and glutamate were analysed. Glucose

TABLE VIII Measured concentrations of glucose (mmol/kg) in blood, CSF and brain tissue as well as calculated intracellular glucose concentrations in control group and in groups bled to 60 and 45 mm Hg respectively. Means ± S.E. The table also gives the concentration ratios of intracellular fluid to CSF, fluid to blood and CSF to blood respectively.

MABP mm Hg	Blood	CSF	Tissue	I.c.	I.c./CSF	I.c./Blood	CSF/Blood
132 ±3 n=9	7.61 ±0.21	5.13 ±0.13	2.91 ±0.12	3.13 ±0.0	0.61 ±0.04	0.41 ±0.03	0.67 ±0.02
60 n=6	15.3 ±2.1	6.14*** ±0.12	4.91 ±0.47	5.79 * ±0.66	0.94 * ±0.10	0.39 ±0.02	0.44 * ±0.06
45 n=8	23.1* ±3.0	7.03 ±0.48	5.93 ±0.64	6.85 ±0.85	0.96 ±0.08	0.31* ±0.03	0.32 ±0.02

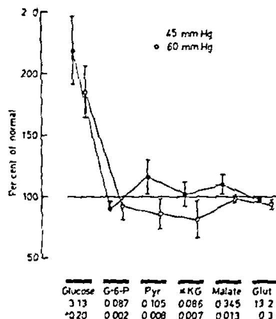


Fig 5 Influence of arterial hypotension upon intracellular concentrations of glucose, G 6 P, pyruvate, α ketoglutarate, malate and glutamate. The values for the hypotensive groups are expressed as per cent of control values which are given below horizontal bars. Means ± S E

was measured in blood, CSF and tissue, whence intracellular concentrations could be calculated. Table VIII shows that the hypotensive conditions were associated with marked increases in the blood glucose concentrations ($p < 0.001$) and by moderate increases ($p < 0.01$) in the CSF glucose concentration. The calculated intracellular glucose concentration was approximately doubled in the 45 mm Hg group. The table also shows that the ratio of the intracellular glucose to the CSF glucose concentration increased, while both the intracellular and the CSF glucose concentrations decreased in relation to the blood glucose concentration. In no single animal was the calculated intracellular glucose concentration lower than 4 mMoles/kg of i c water, and there was thus no indication of glucose depletion.

In order to illustrate the levels of the substrates in the hypotensive groups the values of glucose, G 6-P, α ketoglutarate, malate and glutamate have been compared to the normal values in Fig 5. While glucose increased to more than 200% of the normal value in the 45 mm Hg group, all other substrates were close to normal. In those individual animals in which there were increases in the lactate content there was an increase in malate as well.

Discussion

The present experiments have demonstrated that it is possible to reduce the mean arterial blood pressure of lightly anesthetized rats to 60 and to 45 mm Hg for 30 min without causing any metabolic changes suggestive of an derangement of the cerebral energy metabolism. Thus, a completely unchanged cerebral metabolic state was obtained in 4 out of 6 animals in the 60 mm Hg group, the two remaining

animals showing very small increases in lactate and decreases in energy charge potential when compared to the corresponding ranges in the control group Out of the 8 animals in the 45 mm Hg group there were 2 with increases in lactate and 4 with moderate decreases in ECP However, in all animals the ECP values were within 1% of the mean of the control values and in only two animals was the tissue lactate content higher than 2 mmol/kg Neither the CSF pH nor the intracellular pH showed any significant decrease in the hypotensive groups These results indicate that the dilatation of the cerebral vessels which is known to occur in hypotensive conditions is unrelated to a detectable previous derangement of the energy metabolism or to extra or intracellular acidosis In other words the autoregulation of the cerebral circulation appears to be elicited by other mechanisms than hypoxia and acidosis (see Introduction) The cerebral vasodilatation which occurs in normotensive hypoxemia on the other hand is probably related to lactic acidosis in the tissue (Kogure *et al* 1970 MacMillan and Siesjo 1972 a) therefore different dilatatory mechanisms seem to operate in ischemia and in hypoxemia

The present results have further shown that in hypotension when the tissue lactate is increased there is also a decrease in the energy charge potential and an increase in the calculated cytoplasmatic NADH/NAD ratio The decreases in ECP were small and caused by slight increases in ADP and AMP therefore a change in the energy state could not have been detected if only ATP had been measured In two animals with a decreased ECP value the lactate content was normal (1 44 and 1 34 mmol/kg respectively) It would thus seem that the lactate increase seen in other animals was secondary to a change in the energy state the possible triggering mechanism being the increases in ADP and AMP (Lowry *et al* 1964 Lowry and Passonneau 1966 Atkinson 1968) The triggering mechanism thus appears different from that seen in normotensive hypoxemia during which marked increases in the tissue lactate content occur before there are any detectable changes in ATP ADP or AMP (Siesjo and Nilsson 1971 MacMillan and Siesjo 1972 a)

The present results confirm previous reports from the laboratory (Siesjo and Zwetnow 1970 a and b) in showing an efficient energy homeostasis in the brain even at low perfusion pressures In these experiments moderate elevations were found in the tissue lactate content of many animals at levels of perfusion pressure which were unassociated with detectable changes in ATP ADP or AMP The much less pronounced changes in lactate in the present material may be related to the longer hypotensive period and to the halothane anesthesia (*cf* Nilsson and Siesjo 1970) In contrast to the previous reports the present results have shown that the changes in lactate were accompanied by small increases in ADP and AMP It is probable that such increases can only be resolved with analytical methods of the present sensitivity

In the present experiments no or very slight metabolic changes were observed in the brain at venous P_{O_2} values of about 25 mm Hg In other experiments from the laboratory involving moderate decreases in perfusion pressure after bilateral carotid artery ligation (Eklof and Siesjo 1972) or decreases in blood pressure during

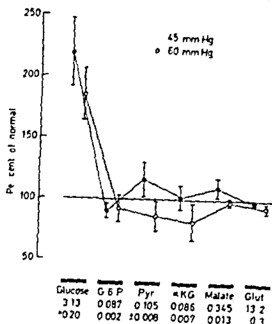


Fig. 5. Influence of arterial hypotension upon intracellular concentrations of glucose, G 6 P, pyruvate, α-ketoglutarate, malate and glutamate. The values for the hypotensive groups are expressed as per cent of control values which are given below horizontal bars. Means ± S.E.

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moderate hypercapnia (Eklof MacMillan and Siesjo 1972 b) a much more pronounced derangement of the cerebral energy state was observed at higher venous oxygen tensions In these experiments tissue acidosis may have contributed in changing unfavorably the flow pattern in the tissue It may therefore be of importance that the present experiments did not lead to tissue acidosis since the widening of the arteriovenous P_{CO_2} difference was compensated by a fall in Pa_{CO_2} and since a marked accumulation of lactate did not occur It should also be recalled that the two animals excluded because of high Pa_{CO} values both showed increased lactate concentrations and decreased ECP values In other word had the Pa_{CO_2} been kept constant hypotension and decrease in CBF should have led to tissue acidosis due to CO retention and it is not certain that such a condition would have allowed an essentially normal energy state at low perfusion pressures The importance of the acidosis will be discussed in a following communication (Eklof MacMillan and Siesjo 1972 b)

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LOWRY O H J V PASSONNEAU F X HASSELBERGER and D W SCHULTZ Effect of

the tissue cells to survive Thus as long as there is some circulation to the tissue oxygen and glucose will be delivered the continuous aerobic and anaerobic metabolism even if insufficient to maintain function and a normal energy state may nevertheless prolong survival of the cells However a continuous anaerobic glycolysis will acidify the tissue and the tissue acidosis by itself is supposed to favour the development of brain edema and thereby hamper reversibility (Lindenberg 1963)

In a preceding communication (Eklöf and Siesjo 1972) we reported experiments in which both carotid arteries were ligated in lightly anesthetized rats either at normal mean blood pressures or after a reduction of the blood pressure to 100 and 70 mm Hg respectively Although carotid artery ligation alone decreased cerebral blood flow to 45—50 % of normal the cerebral energy state as judged from the tissue concentrations of ATP ADP and AMP was not significantly affected When the blood pressure was reduced before the ligation of the carotid arteries there were marked changes in the energy state indicating that the blood flow was reduced to values too low to maintain energy metabolism in the tissue However since the reduction in cerebral venous P_{O_2} was too small to explain the deranged energy state it was concluded that the ischemic model used led to an uneven tissue perfusion and possibly to nonperfusion of localized tissue areas

The present communication deals with the changes in the acid base parameters and in the tissue concentrations of some substrates of the carbohydrate metabolism observed after carotid artery ligation The observations were made on the animals of the preceding communication (Eklof and Siesjö 1972) The objectives of the study was to correlate the degree of acidosis and the extent of substrate depletion to the energy state of the tissue as described in that communication The results also made it possible to evaluate the presence of inhomogeneities in the metabolic response to the reduction of the circulation

Methods

The experiments were identical to those described in the preceding communication (Eklof and Siesjo 1972) The general procedures were as follows Male rats weighing 300—400 g were tracheotomized and artificially ventilated with 70 % N_2O and 30 % O_2 One femoral artery was cannulated for blood pressure recording and for anaerobic sampling of blood and the atlantooccipital membrane was exposed for subsequent sampling of cisternal CSF

There were two series of animals In the first series (A) a burr hole was placed over the superior sagittal sinus and venous blood was sampled for measurements of P_O P_{CO} and pH In this series cisternal CSF was sampled for measurements of either the P_{CO} or total CO_2 content.

In the other series (B) the superior sagittal sinus was not exposed cisternal CSF was sampled for measurements of lactate and pyruvate and a funnel was fitted into a skin incision for freezing the brain *in situ* by pouring liquid nitrogen onto the funnel

In each series there were four groups of animals In one group (control) loose ligatures were placed around the common carotid arteries and the appropriate samples (venous blood and CSF or CSF and tissue) were taken after a 30 min steady state period In the second group the common carotid arteries were ligated and groups of animals were studied either after 5 or after 30 min In the third and fourth groups the mean arterial blood pressure was reduced by means of bleeding to 100 and 0 mm Hg respectively before the carotid arteries were ligated and the animals were studied either after 5 or after 30 min

pH and gas tensions in blood were analysed as described in the preceding communication (Eklof and Siesjo 1972) P_{CO} in cisternal CSF was measured with a micro-CO_2 electro

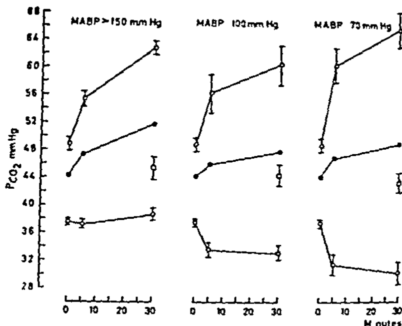


Fig. 1. Relation between arterial P_{CO_2} (lower graphs) and cerebral venous P_{CO_2} (upper graphs) and calculated Pt_{CO_2} (filled circles) at time 0 (control values) and at times 5 min and 30 min, respectively, after bilateral carotid ligation in the normotensive and the two hypotensive groups. The open squares represent P_{CO_2} measured in cisternal CSF at 30 min. Means ± S.E.

(Eschweiler and Co., Kiel) and the total CO_2 content was measured with a microdiffusion method (Siesjö 1962). Lactate and pyruvate in blood and CSF was measured enzymatically (see below). The supratentorial parts of the frozen brains were split in the midline. One hemisphere was used for measurements of the total CO_2 content while the other was analysed for glucose, lactate, pyruvate, α-ketoglutarate and glutamate using methods previously described (Siesjö and Nilsson 1971).

Calculations. The CSF pH was calculated using the pK values given by Mitchell *et al.* (1965) from the total CO_2 content and the P_{CO_2} which was derived by adding the mean difference between the measured CSF P_{CO_2} and the Pa_{CO_2} for the group to each individual Pa_{CO_2} value

$$\text{CSF pH} = \text{pK} + \log\left(\frac{T_{CO_2}}{P_{CO_2} \cdot S} - 1\right)$$

where the solubility factor (S) is 0.0318 mmol/kg × mm Hg (Mitchell *et al.* 1965). Intracellular pH was calculated according to the CO_2 method (see Siesjö and Messeter 1971).

Statistical analysis for significant differences between control group and test groups was performed using Wilcoxon's rank test with the following symbol.

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Results

Acid base changes. In the preceding communication (Eklöf and Siesjö 1972) the blood P_{CO_2} values were described. Fig. 1 shows the measured arterial and cerebral venous CO_2 tensions (open circles), the mean tissue CO_2 tensions (closed circles) which can be calculated by adding 1 mm Hg to the arithmetic means of the arterial and venous tensions (Pontén and Siesjö 1966) and the CO_2 tensions

TABLE I Total CO_2 content in CSF and derived CSF P_{CO_2} (see text) as well as calculated CSF pH in the control group and in the groups with bilateral carotid ligation for 30 min at normotension and hypotension Means ± S E

	Tco mEqv/kg	Pco mm Hg	pH
Control n=6	29 5 ±0 6	45 0 ±1 3	7 42 ±0 01
> 160 mm Hg n=8	28 9 ±0 8	45 1 ±1 9	7 41 ±0 0?
100 mm Hg n=6	27 0 ±0 6	4? 2 ±2 8	7 41 ±0 04
70 mm Hg n=4	?5 7 ±1 1	43 7 ±1 3	7 37 ±0 02

measured in cisternal CSF at 30 min (open squares) The CSF CO tensions were consistently lower than the calculated mean tissue CO tensions probably indicating that cisternal CSF equilibrates with tissue regions which show a lesser degree of reduction in circulation than the supratentorial parts of the brain (see Discussion)

The proposed differences in CO tension between various parts of the brain hamper the derivation of extracellular acid base changes Thus if the measured CSF CO tensions and the measured CSF CO contents are used to calcuate the CSF pH no significant decrease in CSF pH can be calculated for the normotensive group and only a minor decrease in pH is derived for the 70 mm Hg group (Table I) However such a calculation only gives pH in cisternal CSF In the true extracellular fluid of the supratentorial parts of the brain the pH must be lower partly because the P_{CO} was higher (see Fig 1) and partly because the bicarbonate concentration is lower than that of cisternal CSF (see below) Therefore we can only conclude that there must have been an extracellular acidosis already in the normotensive but ligated group but the data do not allow a quantification of the degree of acidosis

Table II shows the lactate and pyruvate concentrations in blood CSF and tissue measured either 5 or 30 min after the ligation of the carotid arteries There was a marked accumulation of lactate in the blood in the hypotensive groups already at 5 min and a smaller and delayed lactacidosis in the CSF In the brain four out of six lactate values in the ligated but normotensive group studied at 30 min were higher than the highest value in the control group Since this increase occurred in spite of an elevated tissue CO tension which by itself would tend to lower the tissue lactate content (see Folbergrova MacMillan and Siesjo 1972) the results indicate that the decrease in CBF was responsible for a moderate lactacidosis in the tissue In the hypotensive groups a marked accumulation of lactate occurred with some individual values exceeding 40 mmol/kg There was a clear disparity between the increases in the tissue and CSF lactate concentrations again emphasizing

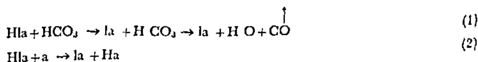
TABLE II Effects of carotid ligation in normotensive and hypotensive rats on the concentration CSF and brain tissue Means ± S E

Experimental group	Blood			CSF
	La	Py	La/Py	La
Controls (n=6)	2 97 ±0 23	0 18 ±0 004	16 9 ±1 0	3 0? ±0 14
Carotid lig 5 min				
> 160 mm Hg (n=6)	4 13 ±0 48	0 19 ±0 02	24 2 ±4 1	4 03 ±0 20
100 mm Hg (n=6)	8 31*** ±1 03	0 23 ±0 02	36 2*** ±4 7	4 81** ±0 22
70 mm Hg (n=6)	10 60*** ±0 98	0 23 ±0 02	45 7** ±2.8	5 18*** ±0 11
Carotid lig 30 min				
> 160 mm Hg (n=6)	4 52 ±0 45	0 20 ±0 01	24 3* ±2 5	4 48 ±0 50
100 mm Hg (n=8)	9 38*** ±1 90	0 22 ±0 02	4? 0*** ±6 1	5 95* ±0 6?
70 mm Hg (n=6)	10 63*** ±0 45	0 22 ±0 16	49.3*** ±5 8	8 08*** ±0 35

probability of differences in flow between the supratentorial and the basal parts of the brain The results indicate that the extracellular lactacidosis in the supratentorial parts of the brain was much more pronounced than the CSF analyses would predict

The proposed inhomogeneity of the extracellular fluid with respect to the HCO_3 concentration makes it difficult to calculate accurately intracellular pH from the CO data Thus since the correction for the extracellular HCO_3 content is based on the measured CSF HCO_3 concentration the calculated pH' will be erroneously low in all situations in which there is a marked lactacidosis in the tissue and thereby a marked decrease in the HCO concentration of interstitial fluid (cf MacMillan and Siesjo 1972 a) We may therefore regard only the values derived for the normotensive groups as being quantitatively correct in showing a significant decrease in pH in the 30 min group partly due to CO retention and partly to a lowering of the intracellular HCO_3 concentration However although it can tentatively be assumed that the CO method would underestimate the pH' in the hypotensive groups the results suggest that the reverse is true Since the results imply that parts of the tissue may be totally ischemic the arguments will be presentd in some detail

When lactic acid is formed in the tissue it will titrate HCO_3 and nonbicarbonate buffers according to the general equations



$$Hla + HCO_3^- \rightarrow la^- + H_2CO_3 \rightarrow la^- + H_2O + CO_2\uparrow \quad (1)$$



$$Hla + a^- \rightarrow la^- + Ha \quad (2)$$

in mmol kg wet tissue) of lactate and pyruvate and on lactate/pyruvate ratios in arterial blood

		Brain		
y	La/Py	La	Py	La/Py
0 14 ±0 01	21 7 ±1 7	1 68 ±0 05	0 09 ±0 005	19 9 ±1 0
0 18 ±0 01	22 6 ±1 1	1 96 ±0 12	0 09 ±0 005	21 4 ±0 8
0 17 ±0 01	28 4 * ±0 9	11 92 ±3 36	0 15 ±0 02	95 5 ±40 3
0 19 ±0 01	28 4** ±1 7	19 60 ** ±1 89	0 15 ±0 03	180* ±62
0 18 ±0 01	25 0 ±2 1	3 15 ±0 96	0 12 ±0 02	24 2 ±3 1
0 19 ±0 01	31 3* ±2 9	20 47 ±6 34	0 14 ±0 02	194 ±82
0 18 ±0 01	46 1* ±2 4	27 44* * ±3 80	0 07 ±0 03	733 * ±227

TABLE III The effects of carotid ligation in normotensive and hypotensive rats on brain tissue concentration of glucose, α ketoglutarate and glutamate (in mmol/kg wet tissue) Means ± S E

Experimental group	Glucose	α KG	Glut
Controls (6)	5 65 ±0 47	0 112 ±0 004	12 31 ±0 24
Carotid lig 5 min 160 mm Hg (6)	5 82 ±0 57	0 131 ±0 01	11 90 ±0 26
100 mm Hg (6)	4 29 ±0 80	0 067 ±0 02	11 90 ±0 42
70 mm Hg (6)	2 31* ±0 14	0 013 ±0 006	11 56 ±0 27
Carotid lig 30 min 160 mm Hg (6)	6 52 ±0 25	0 134 ±0 01	11 56 ±0 35
100 mm Hg (8)	4 50 ±0 64	0 074 ±0 02	10 67 ±0 4
70 mm Hg (6)	1 45* ±0 68	0 011 ±0 01	9 77 ±0 31

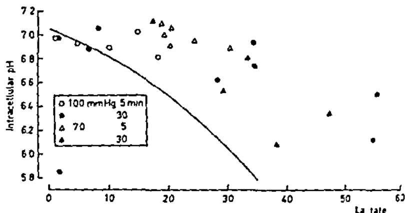
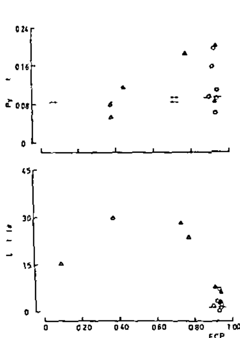


Fig 2 Correlation between brain concentration of lactate (mmol/kg tissue water) and intracellular pH calculated according to the CO_2 method The unbroken line represents the expected variation in calculated intracellular pH with changes in lactate The symbols indicate individual values for the hypotensive animals at 5 min and 30 min after carotid ligation respectively

where a denotes any nonbicarbonate buffer anion which can combine with H at physiological pH values If CO_2 can leave the tissue i e if the tissue is perfused with blood the CO content will decrease by an amount that depends on the amount of lactic acid formed and on the concentration of nonbicarbonate buffer anions This concentration which determines the buffer capacity of the tissue to CO is approximately known (Siesjo *et al* 1972) and it is therefore possible to calculate the expected decrease in pH_i' for any given concentration of lactic acid In the absence of information on the extracellular lactate concentration we can arrive at the approximate cellular concentrations of lactate by assuming that lactate is distributed equally in the tissue water (79 % of the tissue weight) In Fig 2 the unbroken line shows the expected decrease in pH for increases in lactate of from 0 to 35 mmol/kg as calculated from the computor solved model (Siesjo *et al* 1972) The figure also shows that in all cases but one the markedly lactacidotic brains showed a calculated pH' which was much higher than that predicted In the exceptional case the CO pH as calculated for one hemisphere was below 6 although the lactate content of the other hemisphere was only 2 mMoles/kg This finding suggests that the experimental model may give rise to gross inhomogeneities in flow between the two hemispheres However the systematic and large differences between the calculated and expected pH values in all the other experiments require another explanation In all probability these differences were due to the fact that parts of the tissue became totally ischemic and that although the lactic acid formed titrated HCO_3, the gaseous CO formed could not leave the tissue in the absence of a circulation In the calculation of pH' this retained CO will appear as HCO_3 and the derived pH' will be erroneously high (see Discussion)



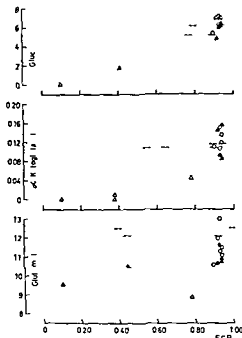


Fig 3

Fig 4

Fig 3 Correlation between the energy charge potential (ECP) and the brain tissue concentrations (mmol/kg wet tissue) of pyruvate and lactate Broken lines indicate mean ± S E for the control values while the symbols represent individual values measured 30 min after bilateral carotid artery ligation open circles in normotension filled triangles in animals bled to 100 mm Hg and open triangles in animals bled to 70 mm Hg

Fig 4 Correlation between the energy charge potential and the brain tissue concentrations (mmol/kg wet tissue) of glucose α ketoglutarate and glutamate For explanation of symbols see Fig 3

Substrate changes

Table III shows the mean values for glucose α ketoglutarate and glutamate In the ligated but normotensive groups the tissue glucose concentrations were either normal or slightly elevated However in the hypotensive groups many animals had low glucose concentrations and some brains were virtually depleted of glucose The same pattern was observed for α ketoglutarate which fell to low values in the 70 mm Hg groups The glutamate values fell inconsistently in the 5 min groups but at 30 min the hypotensive groups showed a clear reduction in glutamate The fall in glutamate in ischemic conditions has not been observed previously (see Maker and Lehrer 1971)

The energy charge potentials (ECP) of the adenine nucleotide system were described in the previous communication (Eklof and Siesjo 1972) In the hypotensive groups there were many grossly lowered ECP values and such values were always associated with high lactate contents (Fig 3) This figure also gives the correspon

ing changes in the pyruvate concentration indicating an increased glycolysis in the occluded normotensive animals and a fall in pyruvate when the ECP is reduced in hypotension Fig 4 shows a correlation between on one hand the energy charge potentials and on the other the tissue concentrations of glucose α ketoglutarate and glutamate The figure shows that when the ECP was decreased below 0 90 signifying a relatively marked change in the tissue contents of ATP, ADP and AMP there were marked decreases in glucose α ketoglutarate and glutamate The results indicate that a derangement of the energy charge potentials was associated with depletion of substrates like glucose and α ketoglutarate

Discussion

The experimental model used in the preceding and in the present communication was chosen to provide a variable degree of cerebral ischemia allowing a correlation of cerebral blood flow cerebral energy metabolism and cerebral venous P_O In the preceding communication (Eklöf and Siesjo 1972) we obtained results which indicated that although the cerebral blood flow, as calculated from arteriovenous oxygen and carbon dioxide differences was reduced to about 50 % of normal by carotid artery ligation alone there were no significant changes in the tissue contents of ATP ADP and AMP However in view of the fact that the spectrophotometric methods used to measure the adenine nucleotides may not be sensitive enough to resolve small changes in the energy charge potentials (see Eklof MacMillan and Siesjo 1972 a) and in view of the small changes in the tissue lactate concentration observed presently we must conclude that carotid artery ligation alone may lead to small changes in the energy state of the tissue

The pronounced variability in results obtained in the 100 mmHg group in itself hampers a correlation between the degree of reduction in flow and the degree of interference with the energy metabolism at last as long as available methods do not allow measurements of flow and energy state in one and the same animal However an even greater obstacle to such a correlation is the inhomogeneity of flow and of energy state suggested by the present results Since this proposed inhomogeneity may apply to most if not all ischemic situations it seems warranted to summarize the evidence on which the hypothesis is based

In a high flow hypoxic situation like hypoxemia the cerebral venous P_{O_2} can be reduced to values around 10 mm Hg without causing any or only very small changes in the energy state of the tissue (MacMillan and Siesjo 1972 b) These results suggest that provided all capillaries are perfused only a very low venous P_{O_2} is required to maintain the oxidative metabolism of the tissue at a level which can keep up a near normal or a normal energy state In a low flow hypoxic situation such as the encountered with the present model a gross derangement of the energy state was observed at venous P_O values of 25—30 mm Hg It seems logical to assume that this dissociation between energy state and venous P_{O_2} is caused by an inhomogenous decrease in the regional flow in the tissue Thus if some tissue areas

are adequately perfused they will heavily influence the P_O of mixed venous blood If there are other areas with a very low perfusion or with no perfusion at all these areas will contribute to the deranged energy state as measured on the tissue as a whole but they will not have a proportional influence on the venous P_{O_2}

It seems essential to decide whether or not the flow ceases altogether in some parts of the tissue or if a sluggish or perhaps intermittent circulation is maintained even in the anoxic areas The dissociation between the expected pH_i values and the values derived with the CO method supports the no flow alternative but the evidence is far from conclusive The evidence is based on the fact that in high flow hypoxic situations like asphyxia (Kaasik Nilsson and Siesjö 1970) or hypoxemia (MacMillan and Siesjo 1972 b) the lactic acid formed in the tissue will titrate intracellular HCO_3 which leaves the tissue via the circulation as CO Based on such experiments and on computor analyses of the intracellular buffer capacity (Siesjo and Messeter 1971 Siesjö *et al* 1972) it may be deduced that if the intra cellular lactic acid concentration increases above 25 mMoles/kg virtually no intra cellular HCO_3 should be left in the tissue In the present experiments many animals showed an excessive lactate accumulation with only a moderate decrease in the HCO_3 concentration of the tissue These results can mean either that lactic acid is formed only in some parts of the tissue or that the CO liberated from HCO_3 cannot leave the unperfused tissue areas If the first of these two alternatives is true the results support the concept of gross inhomogeneity but non perfusion is not necessarily involved However since a few animals showed a metabolic picture suggestive of complete ischemia and since the total CO contents of these hemi spheres were relatively high in spite of lactate contents of 30—45 mmol/kg it *seems realistic to assume that regional no flow is part of the picture in the under* perfused tissue

The present results do not allow any conclusions regarding the nature of the proposed inhomogeneity *i e* whether it involves the microcirculation or relatively large tissue regions One of the present experimental animals showed a metabolic picture which suggested gross inhomogeneity between the two hemispheres and the dissociation between the calculated tissue CO tension and the measured CSF CO tension indicates that a larger reduction in flow occurred in the supratentorial then in the infratentorial parts of the brain It has also been repeatedly pointed out that brain ischemia may preferentially involve the boundary zones between the distribu tion territories of the major cerebral arteries (Lindenberg 1963 Brierley *et al* 1969) Such results as well as those obtained presently (see also Eklof MacMillan and Siesjo 1972 b) emphasize the need for regional methods to study flow and metabolism in the ischemic brain

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glands were therefore used to extend the investigation in the following way The development of the phenomenon was studied Furthermore the effects of repeated injections of 6 hydroxydopamine on the choline acetyltransferase activity was investigated This interesting compound has been found to produce a chemical sympathectomy which in many respects resembles that produced by surgical sympathectomy (Thoenen and Tranzer 1968 Tranzer and Thoenen 1968)

Methods

The experimental animals were dogs (weighing 5 6—11 5 kg) and rats (185—410 g) of both sexes and male golden hamsters (80—165 g) guinea pigs (500—680 g) and mice (33—41 g) *Surgical sympathectomy* In 12 dogs 15 golden hamsters 8 guinea pigs 4? mice and 37 rats the superior cervical ganglion was excised on one side The operations were performed in ether anesthesia after induction with sodium thiopentone 30 mg/kg i v (dogs) or in ether only (golden hamsters mice guinea pigs and rats)

At a varying time after the ganglionectomy (6—47 days usually 30 days) the animals were killed the dogs with an overdose of thiopentone the others in ether

Both the denervated and the contralateral not denervated gland were immediately removed, cleaned in saline and weighed The not denervated glands served as controls Submaxillary glands (golden hamsters guinea pigs mice and rats) parotid glands (dogs and rats) and sublingual glands (rats) were used Owing to the small size of salivary glands of mice and rats the glands from 2 or 3 animals were pooled together in each observation

Chemical sympathectomy 6 hydroxydopamine chloride 100 mg/kg was injected at intervals of one week (altogether 4 times) as recommended by de Champlain (1971) into the tail vein of 10 male rats On the 28th day after the treatment had started the animals were killed in ether The submaxillary (?0) and the sublingual (8) glands were then handled as mentioned above The submaxillary glands from 1 animal and the sublingual from 2 animals were pooled respectively The submaxillary glands of 10 corresponding litter mates and the sublingual glands of 4 corresponding litter mates were used as controls

Choline acetyltransferase determination The glands of the surgically and the chemically sympathectomized animals were then prepared for the analysis of their choline acetyltransferase content The acetone dried powders of the parotid gland of the dog and the rat and the submaxillary glands of the rat were made up in cysteine saline in a concentration of 50 mg/ml the other glands in a concentration of 25 mg/ml Of the tissue extract 0 4 ml from the parotid gland of the dog and 0 2 ml from the other glands were incubated for 1 h at 38 C using the method of Hebb (Nordenfelt 1963) for the determination of the choline acetyltransferase activity The incubate was assayed for acetylcholine on the eserinized frog rectus The enzyme activity was expressed as the amount of acetylcholine synthesized during 1 h per whole gland or pooled glands (the total activity) and per g acetone powder (the concentration)

When the statistical analyses were carried out the denervated gland was always paired with the contralateral not denervated gland of the same animal The glands of the chemically sympathectomized rat were paired with those of its corresponding litter mat All the glands were treated as a group (except for those submaxillaries which were used to study the development of the enzyme increase) although in some cases the time after denervation varied Student s t test was used

The normal variation in choline acetyltransferase activity between right and left submaxillary glands was estimated in 6 golden hamsters and 8 mice

Results

The effects of excision of the superior cervical ganglion and treatment with 6 hydroxydopamine upon the gland weights the total activity of choline acetyltransferase (µg ACh/h/gland or pooled glands) and the concentration of choline acetyltransferase (µg ACh/h/g acetone powder) are shown in Table I II and III and in Fig 1

TABLE I Choline acetyltransferase activity and weight of the parotid and the sublingual glands of the rat (n = 5 3 glands pooled in each observation) after excision of the superior cervical ganglion on one side and the choline acetyltransferase activity and weight of the submaxillary gland of the rat (n = 10 2 glands pooled in each observation) after treatment with 6 hydroxydopamine (6 OH DA) Values are mean ± S E The mean values per gland are given in brackets

	Gland weights (mg)	D/C percentag	Enzyme activity in μg ACh/h/pooled glands	D C percentage	Enzym activity in μg ACh/h g acetone powder	D C percentage
Parotid						
Denervated	471 ± 28 (157)	91 ± 2 7	56 2 ± 3 5 (18 7)	100 ± 3 5	670 ± 30	106 ± 4 9
Contralateral	517 ± 30 (172)		56 2 ± 2 8 (18 7)		633 ± 21	
Sublingual						
Denervated	166 ± 8 (55)	99 ± 1 2	17 0 ± 0 9 (5 7)	104 ± 4 0	650 ± 54	104 ± 2 2
Contralateral	167 ± 7 (56)		16 5 ± 1 5 (5 5)		630 ± 57	
Submaxillary						
6 OH DA treated	420 ± 9 (210)	100 ± 3 0	36 6 ± 1 4 (18 3)	140 ± 8 4	530 ± 27	148 ± 6 9[a]
Controls	423 ± 10 (212)		26 7 ± 1 4 (13 3)		362 ± 14	

< 0 05 [a] < 0 001

TABLE II Choline acetyltransferase activity and weight of the parotid gland of the dog (n = 12) after excision of the superior cervical ganglion on one side Values are mean ± S E

	Gland weight (g)	D/C percentage	Enzyme activity in μg ACh/h/gland	D/C percentage	Enzyme activity in μg ACh/h g aceton powde	D C percentage
Denervated	2 5 ± 0 5	97 ± 1 7	136 9 ± 11 7	124 ± 4 4	307 ± 26	133 ± 5 4
Contralateral	2 6 ± 0 1		112 1 ± 10 9		237 ± 22	

< 0 001

The salivary glands of the rat The total enzyme activity was unchanged in the parotid and sublingual glands of 15 male rats surgically denervated 30 days earlier (Table I)

A gradual increase of the total enzyme activity in the submaxillary glands was shown to begin after about 15 days At the same time the denervated glands seem to gain in weight (Fig 1)

TABLE III Choline acetyltransferase activity and weight of the submaxillary gland of the golden hamster (n = 15) the guinea pig (n = 8) and the mouse (n = 16 2 or 3 glands pooled in each observation) after excision of the superior cervical ganglion on one side Values are mean ± S E In case of pooled observations the mean values per gland are given in brackets

Species	Gland weight (mg)	D/C percentage	Enzyme activity in μg ACh/h/gland	D/C percentage	Enzyme activity in μg ACh/h/g acetone powder	D C percentage
Golden hamster						
Denervated	228 ± 8	103 ± 1.7	66.2 ± 3.4	112 ± 3.1[2]	1 491 ± 75	111 ± 3.5[2]
Contralateral	223 ± 10		59.3 ± 2.9		1 361 ± 86	
Guinea pig						
Denervated	231 ± 11	87 ± 2.0	38.1 ± 3.0	103 ± 2.7	969 ± 46	129 ± 3.5
Contralateral	265 ± 11		36.8 ± 2.7		755 ± 44	
Mouse						
Denervated	185 ± 15 (70)	95 ± 1.6[1]	30.5 ± 1.5 (11.6)	114 ± 4.7[3]	847 ± 55	129 ± 6.1
Contralateral	194 ± 14 (74)		27.1 ± 1.5 (10.3)		666 ± 41	

[1] < 0.05
[2] < 0.01
[3] < 0.001

In 10 male rats treated with 6 hydroxydopamine the total choline acetyltransferase activity had increased with 40 % ($p < 0.001$) in their submaxillary glands (Table I) No increase in enzyme activity was observed in the sublingual glands The total activity was 88 and 108 in per cent of their control glands

The parotid gland of the dog The parotid glands were examined after a time period varying from 7 to 46 days Already after 7 days an increase in the total choline acetyltransferase activity was seen However the present results do not indicate a significant correlation between activity increase and time Therefore the mean figures are presented only As can be seen in Table II the total enzyme activity of the denervated glands increased with 24 % ($p < 0.001$) In normally innervated parotid glands the right glands have been found to have a total activity which was 98 ± 3.9 % of that of the left glands (Ekstrom and Holmberg 1972)

The submaxillary glands of the golden hamster the guinea pig and the mouse When the glands were examined after 30 days the submaxillaries of the golden hamster and the mouse showed a small but significant increase in total enzyme activity i e with 12 and 14 % respectively ($p < 0.01$) In normally innervated submaxillaries of the golden hamster and mouse the right glands had a total activity which was 99 ± 2.7 % (n = 6) and 102 ± 4.5 % (n = 3 8 pairs of glands pooled 2 or 3 together) respectively of that of the left glands No such increase was observed in the guinea pig s submaxillary gland (Table III)

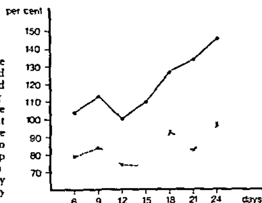


Fig 1 The total choline acetyltransferase activity and dry weight (acetone dried powder) of the rat's submaxillary gland after excision of the superior cervical ganglion The activity and dry weight of the denervated gland is given in per cent of that of the contralateral one 22 female rats were used Each point represents the mean of two observations (in each 2 glands pooled) up to the 15th day thereafter one observation ●—● and ×—× indicate the total activity of choline acetyltransferase and the dry weight respectively

Discussion

Nordenfelt (1964 1965) found that sympathectomy caused an increase of the total choline acetyltransferase activity in submaxillary and parotid glands of cats but not of rabbits This seems to suggest that the phenomenon occurs in some pecies but not in others However the present experiments show that in the same specie one type of gland may show the effect of sympathectomy on the enzyme whereas another type remains unchanged An increase is thus obtained in the parotid but not in the submaxillary gland of the dog The reverse is true in the case of the glands of the rat in this species the enzyme activity of the submaxillary gland is more increased than in any other gland investigated but no change is observed in the parotid gland nor in the sublingual gland

When searching for the mechanism by which sympathectomy may affect the activity of the choline acetyltransferase it seems reasonable to try to connect the different reactions of the glands with differences in their cholinergic or adrenergic innervation Nordenfelt (1964) pointed out that an increase seemed to take place in glands with an initially low concentration of the choline acetyltransferase but not when the concentration was high The present observations on many more glands eem on the whole to substantiate this (Table IV) Thus the activity of the enzyme is low in the parotid but high in the submaxillary gland of the dog high in the parotid and sublingual glands but low in the submaxillary gland of the rat As to the adrenergic innervation there is little information available particularly to make compari ons between glands of different species possible It may be mentioned that in rats the submaxillary gland is well supplied with adrenergic fibres There is for instance a rich supply of secretory nerves The parotid and particularly the sublingual gland of this species are less well supplied with adrenergic nerves (Norberg and Ol son 1965 Ohlin and Perec 1965 Ohlin 1968)

The marked changes obtained in submaxillary glands of rats make these glands convenient preparations when studying the effect of sympathectomy on the cholin acetyltransferase activity Using these glands it was shown that the total e

TABLE IV Glands are arranged after their ability to increase the total activity of choline acetyl transferase (µg ACh/h/gland) after a sympathetic denervation expressed in percent of that of the contralateral (or control) gland The enzyme concentration (µg ACh/h/g acetone powder) of the normally innervated gland is also given

Gland	Increase of total activity in the denervated gland expressed in percent of that of the contralateral or control gland	Enzyme concentration of the normal gland
Rat submaxillary	140[1] 130[3]	367 296 370
Cat parotid	130	123
Dog parotid	124	237
Cat submaxillary	122[2]	277
Mouse submaxillary	114[2]	666
Golden hamster submaxillary	112[2]	1361
Rat sublingual	104[2]	630
Guinea pig submaxillary	103[2]	755
Dog submaxillary	102[2]	748
Rabbit submaxillary	101[3]	983
Rabbit parotid	100[3]	709
Rat parotid	100	633

[1] present study
[2] Nordenfelt (1964 1965)
[3] Ohlin and Perec (1967)

activity increases gradually in the course of some weeks It is of interest to note that at the same time an increase occurs in the cholinesterase activity (Nordenfelt 1968) Further it was found that injections of 6 hydroxydopamine had a similar effect on the enzyme activity in this gland as surgical denervation the sublingual gland was not affected by the two procedures

In some glands degenerating sympathectic nerves thus seem to induce an increase in the activity of the choline acetyltransferase of the cholinergic nerves whether the degeneration is due to surgical or chemical sympathectomy The effect develops gradually and it seems to appear particularly in glands with initial low concentration of the enzyme and perhaps easier when the density of degenerating fibres is high

Nordenfelt (1964 1965) tried to explain the increased enzyme activity as due to collateral sprouting of the postganglionic parasympathetic neurones in the gland No histological examinations have been made on this subject in salivary glands but for a recent report by Garrett (1971) where an increase of cholinesterase staining fibres is shown after sympathectomy of the cat s submaxillary gland Another possibility might be that the increase of choline acetyltransferase occurs within the already existing axonal tissue In the rat submaxillary gland the sympathetic secretory pathway has been shown to be involved in the digestive reflexes (Ohlin 1968) It might be hypothesized that loss of this pathway by surgical or chemical sympathectomy is followed by an increased traffic of secretory impulses in the parasympathetic nerves which might result in an increase of the activity of the transmitter synthesizing enzyme

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Motoneurone Models Based on 'Voltage Clamp Equations' for Peripheral Nerve

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Abstract

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Motoneurone models were developed by modifications of the Frankenhaeuser Huxley equations (1964) for voltage clamp data from the amphibian nerve fibre The major modifications were (i) that the subthreshold sodium inactivation was decreased and (ii) that the Frankenhaeuser Huxley model was provided with a long lasting after hyperpolarization due to potassium permeability changes Besides model versions were developed that also had passive membrane properties (e g a resting membrane time constant) similar to those of spinal motoneurones For various model versions single spikes and the associated afterpotentials were shown in the present paper The main aim was to use the motoneurone models for studies of repetitive impulse firing This will be done in a subsequent paper (Kernell and Sjoholm 1972)

For the interpretation of the function of spinal motoneurones (*e g* their repetitive impulse firing) it is important to know the properties of the motoneurone membrane Thus for instance it would be desirable to know in detail how the membrane permeability to various ions varies with membrane potential and time In certain structures such as nerve fibres data of this kind have been acquired with the aid of the voltage clamp method This technique is however, extremely difficult to apply to a multipolar neurone that is embedded in nervous tissue The technique has been used for spinal motoneurones (Frank Fuortes and Nelson 1959 Araki and Terzuolo 1962) but it has not yet been possible to perform a complete quantitative voltage clamp analysis of the motoneurone membrane A very detailed voltage clamp analysis has earlier been made on the excitable membranes of axons from the squid (Hodgkin and Huxley 1952) and the amphibian *Xenopus laevis* (Frankenhaeuser summary of quantitative data in Frankenhaeuser and Huxley 1964) Mathematical models of the nerve membrane based on voltage clamp data, have been of great use for the understanding of the membrane properties underlying the impulse activity of nerve fibres It seems likely that the membrane of central nerve cells functions according to similar principles as the axonal membrane (*cf* Eccles 1957 Araki and

Terzuolo 1962) Thus the kind of mathematical model that has been formulated for axon membranes (Hodgkin and Huxley 1952 Frankenhaeuser and Huxley 1964) should be useful also for the understanding of the properties of central neurones (cf Stein 1967)

We have been studying neurone models that were based on equations for voltage clamp data from an axonal membrane Our main aim was to compare the functional properties of such neurone models to the properties of a vertebrate nerve cell (the spinal motoneurone) Therefore we choose to base our models on the equation system for a vertebrate axon (Frankenhaeuser and Huxley 1964) instead of using similar data from the squid (Hodgkin and Huxley 1952) We modified the original Frankenhaeuser Huxley model (F—H model) in order to make it conform more closely to certain membrane properties of cat spinal motoneurones (as recorded from the cell body) The most important modifications were

(i) that we decreased the subthreshold sodium inactivation Accommodation to gradually rising currents is typically very slow in undamaged spinal motoneurones (in some cells current ramps as slow as about 0 5 rheobases/s have been tested without any clear evidence for accommodation cf Frank and Fuortes 1960 Bradley and Somjen 1961 Sasaki and Otani 1961 Ushiyama Koizumi and Brooks 1966) This might suggest that the subthreshold sodium inactivation is comparatively slight in undamaged spinal motoneurones (cf Vallbo 1964 Frankenhaeuser and Vallbo 1965)

(ii) that we increased the resting membrane time constant In the standard F—H model the resting membrane time constant is only about 0 066 ms (Frankenhaeuser and Huxley 1964) whereas in a fast spinal motoneurone the resting membrane time constant is usually of the order of 5 ms (e g Burke 1968)

(iii) that we tried to provide the model with afterpotentials such as those of cat spinal motoneurones In a spinal motoneurone a single soma dendrite spike is typically followed by a brief after depolarization (delayed depolarization) (Brock Coombs and Eccles 1952 Granit Kernell and Smith 1963 Kernell 1964) which is succeeded by a prominent after hyperpolarization with a duration of about 50—200 ms and a peak size that often is of the order of 5 mV (Brock et al 1952 Eccles Eccles and Lundberg 1958 Kuno 1959) Experiments have indicated that the after hyperpolarization is predominantly due to a prolonged increase of potassium permeability (Coombs Eccles and Fatt 1955) During much of the duration of the after hyperpolarization there is an approximately exponential decay of the associated conductance change (Baldissera and Gustafsson 1971) There is much evidence indicating that the after hyperpolarization is of particular importance for the repetitive impulse firing of spinal motoneurones (e g Eccles 1936 1953 Pitts 1943 Eccles et al 1958 Kernell 1965 1968 Baldissera and Gustafsson 1971)

It would be of interest to know to what an extent the different membrane properties of axons and nerve cells respectively might be due to simple quantitative variations Therefore we tried as far as possible to modify the original F—H model by chan constants in the various equations rather than by changing the equations t

Furthermore in the present investigation we have avoided to introduce the geometric complexities of spinal motoneurones (*e g* dendrites axon) Thus in our models there were no gradients of membrane potential between different membrane areas and all currents were uniformly distributed to the membrane In spinal motoneurones *the membrane properties of the dendrites are not well known Thus for in*stance, it is uncertain to what an extent dendritic membrane areas might contribute to the afterpotentials following a soma dendrite spike (as recorded from the cell body)

In the present paper we will describe in what ways we modified the original F–H model We will show single spikes and the associated afterpotentials of various model versions In a subsequent paper the models will be used for studies of repetitive impulse firing (Kernell and Sjoholm 1972) Preliminary accounts have been published (Kernell and Sjöholm 1971 a b)

Methods

Nomenclature and symbols Membrane potential (V) is given as intracellular potential minus extracellular potential Outward membrane current is positive V is used for potential relative to the resting potential (E_r) Thus $V = E - E_r$ The suffix ∞ is used for indicating the steady state value of a variable at a given membrane potential (*e g* m_∞ h_∞)

$[Na]_o$ $[Na]_i$ $[K]_o$ $[K]_i$	outside and inside concentrations of sodium and potassium
C	membrane capacitance
g_l	leak conductance
V_l E_L	leak current equilibrium potential
I_L	leak current
I_c	capacity current
I_a	applied current (e g stimulating current)
P_{Na}	sodium permeability
P_p	non specific permeability (partly sodium) also referred to as p-permeability
P_K	fast potassium permeability
P_{K_s}	slow potassium permeability
$\bar{P}_{Na}$ $\bar{P}_p$ $\bar{P}_{K_f}$ $\bar{P}_{K_s}$	permeability constants as indicated by suffix
I_{Na} I_p I_K I_K	ionic currents as indicated by suffix
I_∞	*net membrane current in steady state*
h	variable for inactivation of sodium permeability
m	variable for activation of sodium permeability
p	variable for activation of non specific permeability
n	variable for activation of fast potassium permeability
q	variable for activation of slow potassium permeability
τ_h τ_m τ_p τ	time constant for variable indicated by suffix
α β	rate constants for h m p n or q as indicated by suffix (e g α_h β_q)
A B C	constants in equations for α and β as indicated by suffix (e g $A_{\alpha h}$ $C_{\beta q}$)
R	gas constant
T	ab olute temperature
F	Faraday s constant (in constant field equation el ewhere F stands for Farad)

The mathematical mod l quations to be computed An extra potassium permeability (P_{K_s}) was added in some of the present c lculations (Fig 7–10) Otherwise the equations were precisely like those of Frankenhaeuser and Huxley (1964)

Ionic permeabilities w re calculated from the equations

$$P_{Na} = \bar{P}_{Na}\, m\, h \quad (1)$$

$$P_p = \bar{P}_p\, p \quad (2)$$

$$P_K = \bar{P}_{K_f}\, n \quad (3)$$

$$P_K = P_K q$$

where $\bar{P}_N$, $\bar{P}_p$, P_{K_1} and P_K are constants. The variables h, m, p, r and q are voltage dependent and they vary between 0 and 1 according to the differential equation

$$dy/dt = \alpha_y(1-y) - \beta_y y$$

where y stands for h, m, p, n or q. The rate constants α and β depend upon membrane potential but not upon time. The potential-dependence of α_h and β_h was described by the equations

$$\alpha_h = A(V-B)/(\exp((V-B)/C)-1)$$

$$\beta_h = A/(\exp((B-V)/C)-1)$$

For other αs and βs the equations were

$$\alpha_y = A(B-V)/(\exp((B-V)/C)-1)$$

$$\beta_y = A(V-B)/(\exp((V-B)/C)-1)$$

where y stands for m, p, n or q. At a given membrane potential, steady state values of h, m, ... are given by $\alpha/(\alpha+\beta)$. The corresponding time constants are equal to $1/(\alpha+\beta)$.

The leak current was calculated from the equation

$$I_L = g_L(V - V_L)$$

and other ionic currents were calculated by the aid of the constant field equation. Thus

$$I_N = P_N \frac{EF^2}{RT} \frac{[Na]_o - [Na]_i \exp(EF/RT)}{1-\exp(EF/RT)} \quad (11)$$

$$I_K = P_K \frac{EF^2}{RT} \frac{[K]_o - [K]_i \exp(EF/RT)}{1-\exp(EF/RT)}$$

The value of I_p was calculated from P_p by an equation corresponding to eq. 11 and the value of I_{K_1} was calculated from P_{K_1} by an equation corresponding to eq. 12. The capacity current and the rate of change of membrane potential were calculated from the equations

$$I_c = I_s - (I_N + I_p + I_K + I_{K_1} + I_L) \quad (13)$$

$$dV/dt = I_c/C_m \quad (14)$$

Steady state currents. At a given membrane potential steady state values for the currents I_N, I_p, I_K and I_K could be calculated by the aid of the corresponding steady state values for h, m, p, n and q. At a given membrane potential the net steady current through the membrane (I_∞) was thus

$$I_\infty = I_{N\infty} + I_{p\infty} + I_{K\infty} + I_{K\infty} + I_L \quad (15)$$

In the standard model of Frankenhaeuser and Huxley (1964) the $I_\infty - V$ curve has a positive slope throughout (Fig. 4, line a) and experiments have indicated that this is the case for spinal motoneurones as well (Coombs et al. 1955, Araki and Terzuolo 1962). For model versions that were to be used in our subsequent studies of repetitive impulse firing (Kernell and Sjöholm 1972) we required that the slope of the $I_\infty - V$ curve should be positive throughout (I_∞ determined at 1 mV steps).

Leak current equilibrium potential. The equilibrium potential for the leak current (V_L) was in each model version assigned a value that made I_∞ equal to zero at resting membrane potential (cf. Frankenhaeuser and Huxley 1964). In all computations the resting membrane potential (E) was −70 mV. The value of V_L was usually between +0.025 and −0.022 mV.

Membrane conductance. Membrane slope conductance was computed by calculating the increment of ionic current that would be produced by a positive pulse of 1 mV in the presence of unchanged membrane permeability. Separate calculations were made for each permeability by the aid of the constant field equation (eq. 11 to 12). Total membrane conductance was the sum of g_L and the conductances computed from P_N, P_p, P_K and P_K respectively.

Threshold potential. The threshold potential for spike initiation was defined as the largest depolarization that could be produced by a current pulse of 1 ms duration in the absence of spike firing. In these determinations the difference between the maximum subthreshold and the minimum suprathreshold current was 1% or less.

Units for current, capacitance, conductance and permeability. In the original F−H model current is given as mA/cm², capacitance as F/cm², conductance as mho/cm² and permeability as cm/s (Frankenhaeuser and Huxley 1964). In some of the present model versions current, capacitance, conductance and permeability will be given as values per total cell membrane area instead of as values per cm² of membrane. Thus, in motoneurone-like model versions (e.g. mod. ls Mo 1 and Mo 2, Table II−III) current will be given as nA, capacitance as F, conductance as mho and permeability as cm³/s (i.e. cm/s multiplied by the area of the cell membrane). Values expressed in the latter way are directly comparable to experimental measurements from spinal motoneurones.

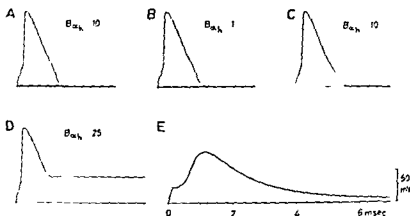


Fig 1 Computed action potentials (membrane potential plotted versus time) Resting membrane potential indicated by horizontal bars with time marks A Standard Frankenhaeuser Huxley model (F—H model) Data for computations Table I—III B—D F—H models with values of B_{α_h} (mV) as indicated E F—H model with standard value of B_{α_h} (—10 mV) but with C_m increased to 151 5 μF/cm which is 75 75 times the standard value The rectangular stimulus pulse was 0 85 mA/cm in A—D and 30 0 mA/cm in E Stimulus duration was 0 12 ms in all cases Same potential and time calibration for A—E

Methods of computation The computations were made on a digital computer at Stockholms Datamaskincentral (IBM 360 75) The integration method was a modified two-step Heun Runge Kutta method devised by A F Huxley (see description in Bromm and Frankenhaeuser 1968) It was controlled that this method gave virtually equal results as the ordinary fourth-order Runge Kutta method (e g Hamming 1962) The integration steps were often made to vary with the membrane time constant It was repeatedly checked that briefer steps gave practically the same results as the steps ordinarily used

Results

Standard F—H model The action potential of the standard model of Frankenhaeuser and Huxley (1964) is shown in Fig 1 A Only negligible afterpotentials followed the spike (cf Frankenhaeuser and Huxley 1964 Frankenhaeuser 1965) The threshold potential was about 19 9 mV (cf p 549) The slope of the $I_\infty—V$ curve was positive throughout (Fig 4 line *a*) For comparison to other model versions standard data of Frankenhaeuser and Huxley (1964) are shown in Table I—III (F—H) and steady state values and time constants for the variables *h m p* and *n* are plotted against membrane potential in Fig 2

F—H model with decreased subthreshold sodium inactivation In the study by Frankenhaeuser and Vallbo (1965 cf also Stein 1967) sodium inactivation was simply altered by changes in B_{α_h} A change in B_{α_h} affected τ_h (Fig 3B) and produced an essentially parallel shift of the $h_\infty—V$ curve (Fig 3A Frankenhaeuser and Vallbo 1965) If the $h_\infty—V$ curve was shifted too much to the right the $I_\infty—V$ curve developed a region of negative slope (Fig 4 lines *c—d*) Such a negative slope appeared with B_{α_h} = 0 mV but not with B_{α_h} = — 1 mV (Fig 4 line *b*) The change of B_{α_h}

TABLE I Constants in equations for α and β (eq 6–9) for various illustrated model versions

Model (illustration)	Variable	α A	α B (mV)	α C (mV)	β A	β B (mV)	β C (mV)
F–H	h	0.1	−10	6	4.5	45	10
(Fig 1A 2)	m	0.36	22	3	0.4	13	20
	n	0.02	35	10	0.05	10	10
	p	0.006	40	10	0.09	−25	20
Mo 1	h	0.8	16	3	4.0	21	1
(Fig 5A–B	m	0.6	22	3	0.6	13	20
6A–B 7)	n	0.16	15	2	0.3[illegible]	−10	10
Mo 2	h	0.8	7	3	4	11	0.2
(Fig 5C–D	m	2	10	0.2	0.6	1	20
6C–D)	n	0.06	9	0.1	0.12	12	0.4
(Fig 7G–I)	p	0.8	45	2	0.00095	10	400
(Fig 7D–I)	q	0.045	45	2	0.00008	10	400

Values of A_{β_h} given in ms⁻ All other values of A given in ms⁻ mV⁻

TABLE II Passive properties of various illustrated model versions

Model (Illustration)	Temperature (C)	C_m	g_L	$[Na]_i$ (mM)	[Na] (mM)	$[K]_i$ (mM)	[K] (mM)
F–H (Fig 1 4)	20	2×10^{-6} (F/cm)	30.3×10 (mho cm)	13.74	114.5	120	2.5
Mo 1 Mo 2 (Fig 5 7 9 10)	38	$5 \times 10^{-}$ (F)	$1 \times 10^{-}$ (mho)	15	150	150	5.5

TABLE III Permeability constants of various illustrated model versions

Model (illustration)	$\overline{P_{Na}}$	$\overline{P_p}$	P_K	Units
F–H (Fig 1A)	8	0.54	1.[illegible]	× 10 cm
Mo 1 (Fig 5A–B 7)	2	—	0.38	10 cm s
Mo 2 (Fig 5C–D)	0.6	—	0.42	10^{-4} m s

from −10 to −1 mV did not markedly affect the shape or size of a single action potential (Fig 1 A–B) The threshold potential was about 19.45 mV for the model version of Fig 1 B i e it was similar to that for the standard F–H model

Fig 1 C demonstrates that a spike might look quite normal even if the corresponding $I_\infty - V$ curve (line c in Fig 4) has a region of negative slope and crosses the voltage axis more than once (i e even if another membrane potential that the resting potential could be maintained at zero membrane current) Thus the spike configuration does not necessarily reveal whether the $I_\infty - V$ relation is abnormal or not Al

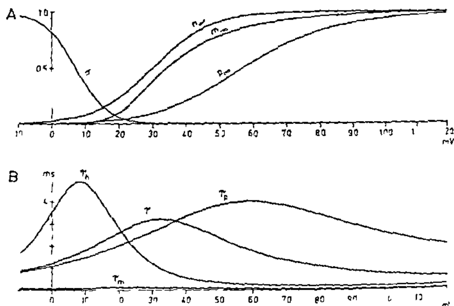


Fig 2 Standard F—H model Steady state values (A) and time constants (B) for variables h m p and n plotted versus membrane potential Data for computations Table I In this and subsequent illustrations membrane potential (V) is given relative to resting membrane potential

Fig 3 F—H model Steady state values (A) and time constants (B) for variable h plotted versus membrane potential for different values of B_{0h} (mV)

Fig 4 Relation between net membrane current in steady state (I_∞ mA/cm²) and membrane potential (mV) for standard F—H model (a) and for F—H models with B_h equal to -1 (b) $+10$ (c) and $+25$ (d) mV

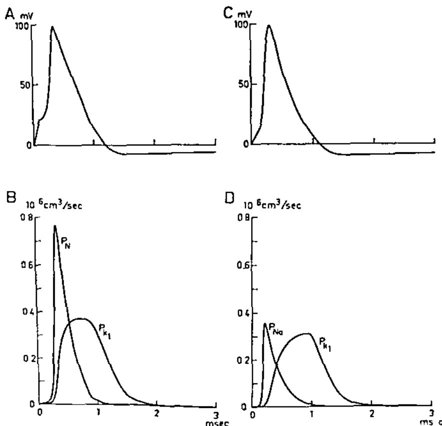


Fig 5 Computed action potentials (plots of membrane potential ersus t me) for models Mo 1 (A) and Mo 2 (C) and the associated permeab l ty cha iges (shown in B for Mo 1 nd n D for Mo 2) For the illustrated model ers ons P_K and P_p were zero Stimulation rectangular current pulses 1000 nA and 0 1 m fo A—B 540 nA and 0 12 ms fo C—D Data for omputations Table I—III T me marks at 1 m interv ls in all di grams At the time of max mum ate of e of the act on potential m mbrane conductance was $140\times10^{-}$ mho for A—B and $100\times10^{-}$ mho fo C—D R st ng membrane conductance $1\ 0\times10^{-}$ mho n both cases Max mum rat of se of action potential 922 V/ for A and 1108 V/s for C

tions giving very drastic changes in the $I_\infty - V$ curve (e g Fig 4 line *d*) could of course lead to abnormal spikes (Fig 1 D note plateau after spike membrane potential could remain indefinitely at this level where I_∞ was zero)

Problems associated with the long membrane time constant of motoneurone like models In a motoneurone like model the resting membrane time constant should be about 5 ms (*cf* Burke 1968) This is about 76 times longer than the resting membrane time constant of the standard F—H model (*cf* values for C_m and g_L in Table II) If membrane capacitance was increased about 76 times the spike of the F—H model became small and extremely slow (Fig 1 E) Compared to the standard F—H model a larger sodium current was needed in models with a resting membrane time constant of ,

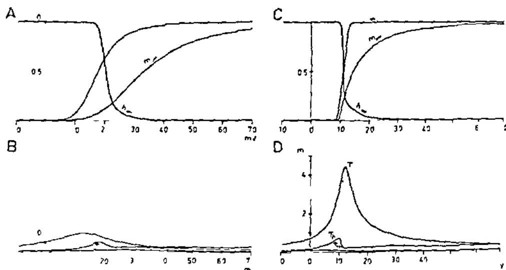
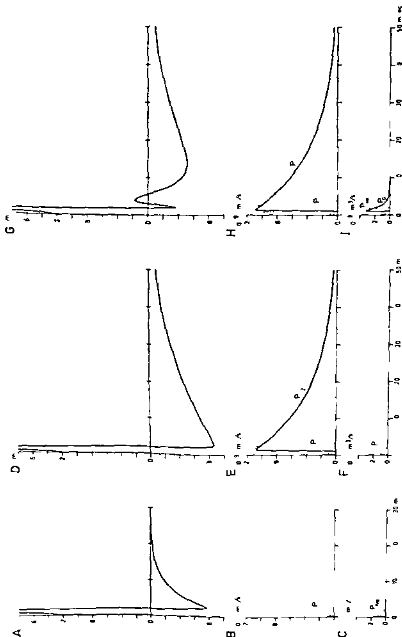


Fig 6 Steady state values (A C) and time constants (B D) for variables h m and n plotted versus membrane potential for model versions Mo 1 (A—B) and Mo 2 (C—D) Data for computations Table 1 In B the curves for τ_h and τ cross at about 40 mV

ms to produce a reasonable rate of rise of the action potential and an appreciable potassium current was necessary for producing anything like a normal fall of the spike In the standard F—H model the fall of the spike is relatively little influenced by potassium permeability (Frankenhaeuser and Huxley 1964)

The large sodium and potassium currents needed in models with a long resting membrane time constant must of course be produced by large permeability changes (P_{Na} or P_K) At a given membrane potential (giving the value of I_L see eq 10) the sodium current required for a certain rate of rise of membrane potential could be computed by combining eq 13 and 14 (p 549) The corresponding sodium permeability (eq 11) and the resulting change of membrane conductance (p 549) were easily computed In a motoneurone like model version with C_m/g_L equal to a membrane time constant of 5 ms a conductance increase due to P_{Na} of at least 46 2 times the leak conductance (g_L) was required to produce a rate of rise of 500 V/sec at a membrane potential 50 mV positive to the resting potential (currents I_s I_p I_{g_1} and I_K assumed to be zero throughout steady state value of P_{Na} assumed to be zero at resting membrane potential temperature and ionic concentrations as for model Mo 1 in Table II) Still larger conductance changes would have to occur if there were simultaneous increases of sodium and potassium permeability (cf Fig 5) if the rate of rise of the action potential were faster or if the same rate of rise were produced at a more positive membrane potential During an action potential in the standard F—H model (resting membrane time constant about 0 066 ms) a maximum rate of rise of membrane potential of over 1900 V/s (Frankenhaeuser and Huxley 1964) coincides in time with an increase of membrane conductance up to only about 6—7 times the resting value



F g 7 Computed afterpotent als (membrane potential plotted ersu tim) and the a oc at d p r neab l ty changes for model Mo 1 w th $\bar{P}_p = 0$ and $P_K = 0$ (A–C) with $\bar{P}_p = 0$ and $P_K =$ 0 034 10 cm /s (D–F) and with $\bar{P}_p = 0\ 0033 \times 10$ cm /s and $P_K = 0\ 034 \times 10^{-}$ m /s (G–I) Fo other computation d ta see Table I–III During pike the m mb ne pot nt al and th assoc ted permeabil ty change for sod um (P) and potas um (P_K) shoot outs de the d g ms St mulation rectangula current pulse of 540 nA nd 0 1? ms T me ma ks at 10 ms inter al in all d grams

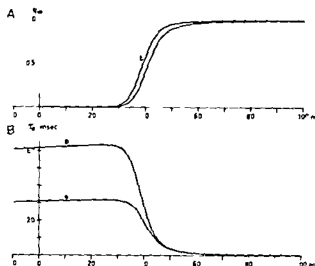


Fig 8 Steady state values (A) and time constants (B) for the variable q plotted versus membrane potential Constants in the equations for the rate constants were as in Fig 7 G (a) and as in Fig 7 G but with B_{β_q} = 0 00004 ms^{-1} mV^{-1} (b) (cf Table I)

Spike generation in motoneurone like model versions (Mo 1 and Mo 2) Motoneurone like model versions were made so that (i) the resting membrane time constant was 5 ms the resting membrane conductance 1×10^{-6} mho (*cf* Table II) and (ii) there was very little subtreshold sodium inactivation (even less than that of the F–H model with $B_{\alpha_h} = -1$ mV) After changes that affected the membrane time constant or the sodium inactivation of the model a number of compensatory adjustments generally had to be made in other equations (*e g* for P_{Na} and P_{K_1}) in order to keep reasonably normal spikes and a normal $I_\infty - V$ curve (positive slope) These adjustments had to be made by a laborious trial and error process that necessitated a great number of computations In this modelling we required that a single spike should have a duration (about 1 ms) and an amplitude (about 90–100 mV) that were similar to values published for cat spinal motoneurones in good condition (Brock *et al* 1952) We did not however bother much about the detailed shape of the spike In these motoneurone like model versions only the sodium permeability (P_{Na}) and the fast potassium permeability (P_{K_1}) were of particular importance for spike shape The other permeabilities (P_p and P_{K_2}) were used for the generation of afterpotentials (see p 557) In the motoneurone like models (Mo 1 and Mo 2) the sodium and potassium concentrations (Table II) were those quoted for cat spinal motoneurones (Eccles 1957 p 24) and temperature in the constant field equation (eq 11–12) was put to 38 C

For 2 motoneurone like model versions the spike generating properties are illustrated in detail in the present paper (models Mo 1 and Mo 2 Fig 5–6) Spikes are shown in Fig 5 A and C and the associated permeability changes are displayed in Fig 5 B and D Data used for the computations are given in Table I–III In model Mo 1 (Fig 5 A–B) only moderate attempts were made to change the potassium or sodium activation and the shape of the $m_\infty - V$ curve (Fig 6 A) was similar to that

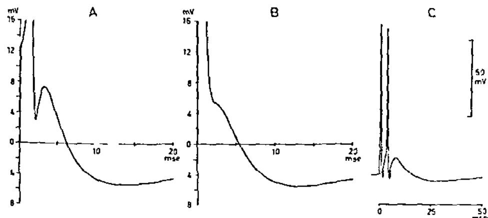


Fig 9 Computed afterpotentials (membrane potential plotted versus time) A Model Mo 1 with data as in Fig 7 G except that $A_{\beta p} = 0.0017$ ms^{-1} mV^{-1} $\bar{P}_q = 0.017 \times 10$ cm^3/s $A_{\alpha q} = 0.03$ ms^{-1} mV and $P_{K_2} = 0.1 \times 10$ cm/s B Model with resting membrane time constant 5 msec but with other sets of data for P_K and P_{K_1} than those valid for models Mo 1 or Mo 2 Data for P_p and P_K as in Fig 7 G C Model Mo 1 with data as in Fig 7 G except that A_{β} = 0 000_5 ms^{-1} mV^{-1} Stimulation Rectangular current pulse of 540 nA and 0 12 ms for A 1000 nA and 0 1? ms for B and 1000 nA and 0 10 ms for C Spikes in A and B shoot outside the diagrams In C the level of resting membrane potential is indicated prior to the onset of stimulation at time zero (see time calibration under potential time plot of C)

of the standard F—H model (Fig 2 A) In model Mo 2 (Fig 5 C—D) the $n_\infty - I$ curve as well as the $m_\infty - I$ curve was very steep (Fig 6 C) Model Mo 2 had a very sharp threshold and very little potassium or sodium permeability was activated at membrane potentials more than 1 mV below threshold The threshold potential was about 13 3 mV for model Mo 1 and 9 8 mV for model Mo 2

Afterpotentials In models Mo 1 and Mo 2 the fast potassium permeability (P_{K_1}) outlasted the spike by about a msec (Fig 5) Consequently the fast potassium permeability caused a brief after hyperpolarization to follow the spike (Figs 5 and 7 A) During most of this after hyperpolarization membrane potential returned passively toward the resting level without further permeability changes (Fig 7 A—C) It proved difficult to change the constants of a single potassium system in such a manner that the fall of the spike as well as the size and time course of the after hyperpolarization were reasonably satisfactory Therefore we chose to use a separate slow potassium permeability change (P_{K_2} dependent on variable q *cf* eq 4) for the production of long lasting after hyperpolarizations The equations for this permeability system were the same as those for the ordinary potassium system of the standard F—H model (p 549)

The potential dependence of the rate constants for the slow potassium system was such that q_∞ was large during a spike but practically zero at subtreshold membrane potential (Fig 8 A *cf* Table I) Thus no activation of the slow potassium permeability occurred below threshold Furthermore τ_q was brief during a spike but v

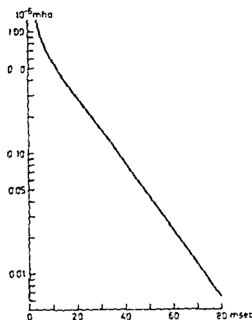


Fig 10 Computed conductance change associated with the afterpotentials of Fig 7 C Semilogarithmic diagram of total membrane conductance minus resting membrane conductance plotted versus time after onset of stimulation.

long and nearly constant at subthreshold membrane potentials (Fig 8 B) Thus the value of q (and thus of P_{K_2}) would increase rapidly during a spike and after the spike the value of q would decay nearly exponentially The time constant for the decay of q (and P_{K_2}) could easily be altered by changing the value of A_{β_q} (Fig 8 B) The extent to which q was increased during a spike could be varied by changes in A_{α_q}. The size of the after hyperpolarization could be varied by changing A_{α_q}, P_K or both Fig 7 D shows an example of an after hyperpolarization produced in model No 1 provided with a slow potassium system Such prolonged after hyperpolarizations could of course be generated in any model version (e g in the standard F–H model No 1 or No 2) by the simple addition of a slow potassium system.

Early in the post-spike period the shape of the afterpotential in Fig 7 D differs from that of spinal motoneurones In motoneurones there is initially a gradual increase of membrane potential until the peak of the after hyperpolarization is reached at about 10–20 ms after the spike (Brock *et al* 1952, Kuno 1959 Kernell 1965) In the present neurone models (e g No 1 or No 2) a similar shape of the afterpotential could be produced with the aid of the non specific permeability system (p-permeability P_p The rate constants of this system were then made to depend on membrane potential in a manner similar to the rate constants for the slow potassium system cf Fig 8 Table I) In Fig 7 G the decay of P_p was slow enough to make the peak of the prolonged after hyperpolarization occur at about 13 ms after spike onset

If the activation of the p-permeability (P_p) was sufficiently strong and long lasting a single spike elicited by a brief stimulus would be followed by a period of

depolarization (delayed depolarization) which gradually merged into the prolonged after hyperpolarization (Fig 7 G and 9 A) In models Mo 1 and Mo 2 there was a marked dip (due to mainly P_{K_1}) between the spike and the delayed depolarization (Fig 7 G and 9 A) The example of Fig 9 B shows a motoneurone like model version (resting membrane time constant 5 ms) which lacked the dip between the spike and the delayed depolarization The particular version of Fig 9 B had however other disadvantages (*e g* more subthreshold sodium inactivation than models Mo 1 and Mo 2)

If the delayed depolarization was very large and long lasting (*i e* large P_p which was not sufficiently counteracted by potassium permeability) it could lead to re excitation of the neurone model (Fig 9 C) Thus in the latter case one brief stimulus would elicit a burst of spikes A somewhat similar behaviour has been observed experimentally for some neurones of the mammalian brain (hippocampus Kandel and Spencer 1961 olivary nucleus Crill 1970)

The prolonged after hyperpolarizations of the present neurone models were largely due to an exponentially decaying potassium permeability Consequently the decay of extra membrane conductance was virtually exponential during most of the afterpotential (Fig 10 computed for the afterpotentials of model Mo 1 in Fig 7 G) At times later than 11 ms in Fig 10 the time constant of decay of conductance was about 16 ms This time constant is within the range of values for fast (large) hindlimb motoneurones (Kernell unpublished) At the peak of the prolonged after hyperpolarization in Fig 7 G the membrane resistance was about 70 % of the resting resistance (Fig 10) Similar values are indicated for motoneurones by the results of experimental studies concerning the effect of injected current on the amplitude of the after hyperpolarization (Coombs *et al* 1955)

Discussion

Below a brief discussion will be given concerning some differences and similarities between the membrane properties of the present motoneurone like models (Mo 1 and Mo 2) and cat spinal motoneurones

Passive properties The resting conductance (1 × 10 • mho) and the resting membrane time constant (5 ms) of models Mo 1 and Mo 2 were made similar to the values for fast (large) hindlimb motoneurones (*cf* Kernell 1966 Burke 1968) The present models do not however include any of the geometrical complexities (dendrites axon) and passive cable properties of spinal motoneurones (*cf* Rall 1970)

Subthreshold rectification Among spinal motoneurones studied at subthreshold membrane potential the rectifying properties seem rather variable In some spinal motoneurones there is apparently very little steady state rectification at subthreshold membrane potential whereas in other cells the steady state membrane conductance might tend to become increased by hyperpolarization and decreased by subthreshold

depolarization ('anomalous rectification) (Coombs *et al* 1955 Nelson and Frank 1967, Baldissera and Gustafsson 1970 concerning the time dependence of some rectifying phenomena, see Ito and Oshima 1965, Nelson and Lux 1970) In models Mo 1 and Mo 2 there was no rectification for hyperpolarizing currents In model Mo 1 the slope of the $I_{\infty}-V$ curve was less steep at −66 to −59 mV than at more negative membrane potentials (resting potential −70 mV) This was however due to subthreshold activation of sodium permeability and not to any anomalous rectification In model Mo 2 there was practically no subthreshold rectification (p 557 *cf* Fig 6 C)

Suprathreshold sodium and potassium activation In the present models the sodium and potassium permeabilities were potential and time dependent Voltage clamp experiments have suggested that this is the case for the soma dendrite membrane of motoneurones as well (Araki and Terzuolo 1962) Such voltage clamp experiments have also suggested that the maximal sodium activation might correspond to an increase of membrane conductance to about 5 5−9 times the resting membrane conductance (Araki and Terzuolo 1962) For theoretical reason this tentative figure seems too small In a motoneurone like model with a resting membrane time constant of 5 ms a sodium permeability which causes the membrane conductance to increase to 9 times the resting conductance would change membrane potential at a rate of less than 100 V/s at 40 mV or more above resting potential (calculations made as on p 554) For spinal motoneurones in good condition the maximum rate of rise of an action potential is at least 300−500 V/s (Brock *et al* 1952)

Spike configuration In models Mo 1 and Mo 2 spike shape differed in various details from that of spinal motoneurones Spike amplitude and duration were made similar to those of spinal motoneurones In the models the spike did not propagate and it occurred in a homogenous membrane In motoneurones the spike shape is influenced by the fact that the spike is first triggered in a low threshold region (IS region A region) which thereafter activates the major portion of the membrane (SD region B region) (Coombs Curtis and Eccles 1957 Fuortes Frank and Becker 1957 Araki and Terzuolo 1962)

Delayed depolarization In versions of models Mo 1 and Mo 2 that were provided with a delayed depolarization a prominent dip was separating the spike and the delayed depolarization (Fig 7 G and 9 A) Usually no such very prominent post spike dip is apparent in cat spinal motoneurones (Brock *et al* 1952 Kernell 1964) but it occurs in other kinds of vertebrate nerve cells (*e g* toad motoneurones Araki Otani and Furukawa 1953) The origin of the delayed depolarization in moto neurones is unclear (see discussions by Brock *et al* 1952 Granit *et al* 1963, Kernell 1964 Nelson and Burke 1967)

After hyperpolarization The peak size of the prolonged after hyperpolarization in Fig 7 G was made similar to that following an antidromic spike in a spinal moto neurone with a resting membrane potential around −70 mV (*cf* Brock *et al* 1952) The time course of the same afterpotential (Fig 7 G) was made relatively similar to that of spinal motoneurones (cf p 558) In some motoneurones (particularly those with a relatively late peak of the after hyperpolarization) the membrane conductance appears to decay less rapidly just before the peak of the after hyperpolarization than later on (Baldissera and Gustafsson 1971) This was not the case for the model of Fig 7 G (see Fig 10) In spinal motoneurones the prolonged after hyperpolarization is typically followed by a small overshoot (late after depolarization Eccles *et al* 1958) whose origin is unclear (*cf* however Ito and Oshima 1965) No such late after depolarization would be expected to occur in the present neurone models In these models (Fig 7) there was an asymptotic return of membrane potential toward the resting level

In a subsequent paper the model versions developed and described in the present paper will be used for studies of repetitive impulse firing (Kernell and Sjoholm 1972)

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Augmented Noradrenaline Release Following Nerve Stimulation after Inhibition of Prostaglandin Synthesis with Indomethacin

By

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Abstract

JUNSTAD M P H CHANH and A WENNMALM *Augmented noradrenaline release following nerve stimulation after inhibition of prostaglandin synthesis with indomethacin* Acta physiol scand 1972 86 563—567

The effect of inhibition of prostaglandin synthesis on the release of noradrenaline induced by sympathetic nerve stimulation was studied in the isolated perfused rabbit heart It was found that indomethacin (Indomeé® Merck Sharp and Dohme) a drug known to inhibit prostaglandin synthesis decreased the outflow of prostaglandins normally induced by nerve stimulation Simultaneously the outflow of noradrenaline in response to nerve stimulation was augmented Since indomethacin neither caused any release of noradrenaline from the heart in the absence of nerve stimulation nor affected the uptake of exogenous noradrenaline it is concluded that the increased noradrenaline release reflects an inhibition of a feed back mechanism using endogenously formed prostaglandins for limitation of noradrenaline release

Prostaglandins of the E series (PGE_1 PGE) have been shown to inhibit the release of noradrenaline (NA) in response to nerve stimulation in various tissues and species (*cf* Hedqvist 1970 Wennmalm 1971) On the basis of these results and since PG s are released from isolated tissues in response to sympathetic nerve stimulation (Gilmore Vane and Wyllie 1968 Davies Horton and Withrington 1968) it has been proposed (Hedqvist 1969) that endogenous prostaglandins inhibit the release of noradrenaline caused by nerve stimulation The existence of an endogenous feed back mechanism for regulation of NA release has also been proposed by other authors (Haggendal 1970 Farnebo and Hamberger 1971) Strong evidence for a prostaglandin mediated control have been obtained after experiments in the isolated perfused sympathetically innervated rabbit heart (Samuelsson and Wennmalm 1971) In this preparation inhibition of prostaglandin synthesis with 5 8 11 14 eicosa tetraynoic acid was accompanied by an increased release of NA in response to sympathetic nerve stimulation Recently these results have been confirmed after experiments in the isolated cat spleen (Hedqvist Stjarne and Wennmalm 1971) in the vas deferens of the rat and the guinea pig (Swedin 1971)

The present paper presents re-examination of the effect of inhibition of prostaglandin synthesis on the release of NA in response to sympathetic nerve stimulation. It has recently been shown (Vane 1971) that the antiflogistic drug indomethacin is capable to inhibit the synthesis of PG:s in lung homogenate. It was therefore of interest to study if inhibition of PG synthesis with a substance of chemical configuration differing from 5, 8, 11, 14-eicosatetraynoic acid was followed by the same type of augmented NA release as that obtained when PG synthesis was inhibited by the acetylenic compound.

Methods

24 rabbits of mixed strains and sexes were used for the study. They were killed by a blow on the head and bled from the left carotid artery. The heart with intact left and right sympathetic nerve supply was dissected out according to Loffelholz and Muscholl (1969). The organ was perfused at a temperature of 37° C and a pressure of about 60 cm H_2O with Tyrode's solution (conc. in mM: NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.6), aerated with 6.5 % CO_2 in O_2. Ascorbic acid 20 µg/ml was added to the solution. The nerves with adjacent tissue were pulled through separate plastic tubes with platinum rings in their walls serving as electrodes and connected to Grass Model S5 stimulators. They were stimulated by rectangular pulse trains of supramaximal strength and 1 ms duration. The apex of the heart was connected to a strain gauge transducer and heart rate and contractile force were recorded on a Grass Model 5 D Polygraph. The effluent from the heart was collected during the nerve stimulations and until the contractile response had faded out. Indomethacin dissolved in 0.1 M phosphate buffer pH 7.2 was infused through a cannula immediately above the aorta to produce final concentrations of 10 M to 7×10^{-5} M.

14 hearts were used for studying the influence of indomethacin on the outflow of NA in response to nerve stimulation. They were stimulated by 3 imp/s for 6 min. The effluent from the heart was collected in 2 min periods and immediately acidified and chilled. In 7 hearts indomethacin was infused from the beginning of the second 2 min effluent collection period and until the end of the stimulation. The effluents were purified on alumina and assayed for NA according to Euler and Lishajko (1961). The ratio outflow of NA during the second or third 2 min period/outflow of NA during the first 2 min period was estimated both for hearts infused with indomethacin and for the controls. The difference between the ratio during the second and third period for hearts infused with indomethacin and the controls was statistically analyzed using Student's t test.

7 hearts were used for testing the effect of indomethacin on the outflow of prostaglandins from the heart on nerve stimulation. 4 of these hearts were initially infused with indomethacin from 3 min before and until the end of the collection of effluent. Effluent was collected during two to three 5 min periods during which the hearts was stimulated at a frequency of 5 imp/s. The stimulation periods were separated by 15 min intervals. From the remaining 3 hearts perfusate was collected similarly but there was no infusion of indomethacin prior to or during the nerve stimulation. The pooled effluents from each heart were adjusted to pH 3 and extracted for lipid twice with equal volumes of ethylacetate. After washing once with 1/20 volume of 0.5 M acetate buffer pH 6.5 and once with 1/20 volume of H_2O the ethylacetate was evaporated to dryness. The residue was dissolved in 3 ml of Tyrode's solution. The extracts were tested for smooth muscle stimulating activity on a superfused rat stomach strip mounted in a 5 ml organ bath.

3 hearts were used to test if indomethacin affects the uptake of exogenous NA in the organ. After a 15 min resting period the heart was perfused for 10 min with a Tyrode's solution containing 5 µCi l NA-³H (Amersham) and 10 µg NA per liter. After this perfusion was continued with NA free Tyrode's solution and indomethacin was infused to produce a final concentration of 5×10^{-5} M for 25 min. After 15 min of indomethacin infusion perfusion was switched to NA-containing Tyrode's solution. During the two periods of perfusion with labelled and unlabelled NA 0.5 ml aliquots of the effluent were taken after 4, 7 and 10 min. They were added to a 7:3 toluene absolute ethanol solution containing 4 g of 2,5-diphenyloxazole and 100 mg 1-4 bis 2(4 methyl-5 phenyloxazolyl) benzene per liter of toluene. The samples were counted in a Packard Tri Carb Liquid Scintillation Spectrometer.

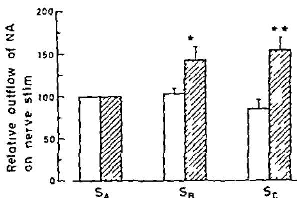


Fig 1 Outflow of NA during 3 consecutive 2 min periods of postganglionic sympathetic nerve stimulation (3 imp/s) expressed as percent of the outflow of NA during the first 2 min period Striped columns Hearts infused with the prostaglandin synthesis blocker indomethacin during the second and third 2 min period

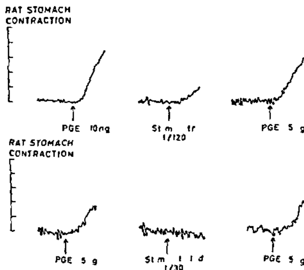


Fig 2 Isolated superfused rat stomach muscle strip Contractile response to fractions of the lipid extract of the perfusate from untreated (Stim extr) and indomethacin treated (Stim extr Ind) hearts Perfusate collected during sympathetic nerve stimulation (3 imp/s)

Results

When the heart was beating spontaneously *i e* during the non stimulation periods only tracer amounts of NA were found in the effluent Infusion of indomethacin did not change the spontaneous outflow of NA from the heart Thus there was no evidence that indomethacin exerted a direct NA releasing effect on the nerve endings in the heart

Stimulation of the sympathetic nerves to the heart for 6 min at a frequency of 3 imp s caused a marked increase in heart rate and contractile force and in addition an outflow of NA which was 137 ± 17 ng (mean ± S E n = 14) during the first 2 min period of stimulation During the following two 2 min periods the outflow of NA was 103 ± 7 % (mean ± S E n = 7) and 85 ± 11 % (n = 7) respecti

the outflow during the first 2 min period When indomethacin was infused during 4 min from the beginning of the second period the outflow of NA induced by nerve stimulation increased to 143 ± 15 % (n = 7) during the second and to 154 ± 15 % (n = 6) during the third 2 min period relative to the first stimulation period (Fig 1) The difference in outflow of NA during the second and third period relative to the first between indomethacin and control experiments is statistically significant (P* < 0 05 and P** < 0 01 respectively)

The lipid extract of the effluent leaving the heart during sympathetic nerve stimulation was tested for biological activity on an isolated rat stomach muscle strip All (n = 4) extracts of the effluents from indomethacin treated hearts entirely lacked biological activity The 3 extracts of the effluents from untreated stimulated hearts all possesseed smooth muscle stimulating activity (Fig 2)

In control experiments 72 ± 4 % (mean ± S E n = 3) of exogenous NA infused appeared in the effluent from the heart When indomethacin was infused from 15 min before and during the second NA infusion 71 ± 4 % (n = 3) of the amine appeared in the effluent Thus there was no evidence that indomethacin blocks the uptake of exogenous NA

Discussion

In the present study it is shown that inhibition of the synthesis of prostaglandins in the isolated perfused sympathetically innervated rabbit heart is followed by an augmented overflow of the adrenergic transmitter NA on nerve stimulation This increased overflow is not caused by a direct effect by indomethacin on the sympathetic nerve endings but a consequence of the inhibition of the endogenous synthesis of prostaglandins This statement is based upon the following fact

1 Infusion of indomethacin in the absence of nerve stimulation did not cause any release of NA nor did it change the mechanical performance of the heart There is thus no reason to believe that indomethacin acts as an indirectly acting sympathomimetic amine

2 Drugs like cocaine imipramine etc are known to increase the overflow of NA from isolated organs on nerve stimulation by blocking the reuptake of transmitter In addition they inhibit the uptake of exogenous NA Indomethacin did not change the ability of the heart to retain exogenously administered NA as shown by the fact that the same amount of labelled NA was found in the perfusate before and after indomethacin treatment Thus there is no reason to assume that indomethacin increases the overflow of NA by decreasing the neuronal reuptake of transmitter

3 Indomethacin in concentrations which augmented the overflow of NA from the heart on nerve stimulation totally abolished the outflow of biologically active lipids from the organ These lipids have recently been identified as PGE_1 and PGE (Samuelsson and Wennmalm 1971)

In a recent paper it has been shown that inhibition of the synthesis of prostaglandins in the rabbit heart by the acetylenic compound 5 8 11, 14 eicosatetraynoic

acid is accompanied by an increased release of NA on nerve stimulation (Samuelsson and Wennmalm 1971) In this paper it was concluded that the enhanced release mirrored inhibition of the prostaglandin mediated endogenous mechanism which normally restricts the release of transmitter It seems highly probable that indo methacin acts in the same way *i e* increases the release of NA on nerve stimula tion *via* inhibition of the endogenous synthesis of prostaglandins

Data are now available which support the concept of such an endogenous mechanism in many different ways Thus evidence have been obtained after experi ments with the rabbit heart the cat spleen and the vas deferens of the rat and guinea pig (for references see introduction) In these studies the endogenous synthesis of prostaglandins was inhibited by 5 8 11 14 eicosatetraynoic acid In the present study a different type of inhibitor of prostaglandin synthesis is used with similar results

It seems feasible to state that the endogenous mechanism for regulation of NA release originally proposed by Hedqvist (1969) can be regarded as fully demon strated However the advantage of such a regulatory mechanism seems still unclear and needs further investigation

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A Paradoxical EEG Response during Combined Hypoxemia and Unilateral Carotid Artery Ligation in the Rat

By

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In an attempt to correlate changes in energy metabolism circulation histology and electroencephalographic activity of the brain in cerebral hypoxia we have combined arterial hypoxemia (Pa_{O_2} 18—30 mm Hg) with unilateral carotid artery ligation in the rat (Salford *et al* 1972) The ligated side has consistently shown metabolic changes indicative of a more serious derangement of the energy metabolism However we have recently found that the EG behaves paradoxically in that the EEG changes are more pronounced on the unligated side Since the results bear on the relationship between energy state and EEG in the hypoxic brain, a preliminary account of the experiments is given

The experiments were performed on male Wistar rats which were lightly anesthetized with 70 % N_2O and 30 % O_2 immobilized and artificially ventilated so as to give a Pa_{CO_2} of 35—40 mm Hg The body temperature was kept close to 37° C When a respiratory steady state was obtained the inspired oxygen tension was reduced to give Pa_{O_2} values in the range 18—30 mm Hg and simultaneously the right common carotid artery was ligated Arterial blood was then sampled at 5 and at 25 min and at 30 min the tissue was frozen *in situ* for subsequent measurements of metabolites using specific enzymatic (fluorometric) techniques (Lowry *et al* 1964 Folbergrova *et al* 1969) It was carefully controlled that the mean arterial blood pressure was maintained above 120 mm Hg and if necessary i v injections of sodium bicarbonate were given Throughout the whole experiments the EEG was recorded from both hemispheres using bipolar technique One vertex electrode and two electrodes placed symmetrically over the parietal area were placed on the periost Two leads from the vertex electrode and the respective parietal electrodes were recorded

Table I shows the parameters used to evaluate the energy state of the tissue The table compares values obtained in a group of ligated animals maintained for 30 min

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TABLE I Bra n tissue concentrations of ATP ADP AMP and lactate (mmol/kg w et tissue) as well as the calculated energy charge potential (ECP) in rats with unilateral carotid artery ligation on the right side (dx) In the hypoxemic group the Pa_O was reduced for 30 min The ECP value was calculated as $\frac{ATP+0\ 5\ ADP}{ATP+ADP+AMP}$

Pa_O mm Hg		ATP	ADP	AMP	ECP	La
117	Dx	3 06	0 278	0 032	0 949	1 72
±9		±0 02	±0 003	±0 001	±0 001	±0 09
	Sin	3 05	0 274	0 030	0 950	1 69
		±0 03	±0 011	±0 001	±0 001	±0 10
20 5	Dx	2 45	0 539	0 274	0 834	19 23
±0 8		±0 20	±0 098	±0 117	±0 049	±1 72
	Sin	3 02	0 373	0 036	0 935	12 98
		±0 03	±0 043	±0 010	±0 008	±1 56

at a Pa_O of 18—24 mm Hg to those measured in ligated but normoxic animals The figures show that a more pronounced lactacidosis developed on the ligated side (dx) of the hypoxemic animals and that the individual adenine nucleotides as well as the calculated energy charge potentials (Atkinson 1968) indicated a more serious derangement of the energy state

Fig 1 shows a typical EEG recording obtained from the 2 hemispheres The normal rat EEG in these conditions consists of a regular 5—6 Hz activity with some low voltage 3 Hz activity superimposed During hypoxia there were changes on both sides of the type usually attributed to cerebral hypoxia (Schmahl *et al* 1966) *i e* both the amount and frequency of the fast activity is reduced and some 1—2 Hz activity appears The amplitudes increases simultaneously However the EEG changes were consistently more marked on the unligated side (sin) and there was thus a dissociation between the energy state and the EEG pattern

The present results have given 2 interesting aspects on the relationship between the energy state of the brain and the EEG in a hypoxic ischemic situation First there were consistent EEG changes of the type usually attributed to cerebral hypoxia on the unligated side of the brain (sin) in spite of the fact that neither the ATP nor the AMP concentrations had changed Thus the biochemical correlates of these EEG changes consisted of a lactic acidosis and of a very moderate change in the energy charge potential caused by a small increase in [ADP] Secondly although the ligated side (dx) showed more pronounced biochemical changes with a clearcut reduction in energy charge potential and an excessive lactic acidosis the EEG was seemingly less altered than on the unligated side

There is no immediate explanation for the paradoxical EEG response observed but the results may possibly be explained in terms of regional ischemia on the side of the ligation Thus since the tissue showed an excessive lactic acidosis and since acidosis seem to predispose to preferential perfusion of some tissue areas (Siesjo Eklof and MacMillan 1972) it is conceivable that areas of no-flow may have existed either in a dissiminated way or in the form of arterial border zone ischemia

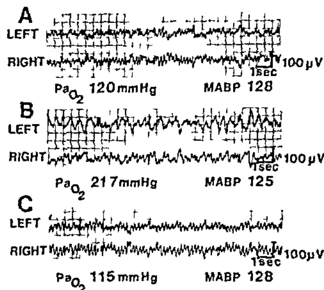


Fig 1 Bipolar EEG recordings from left and right hemispheres of a normotensive rat after ligation of the right common carotid artery A) before reduction of the Pao B) after 15 min of reduced Pao C) 20 min after restitution of the Pao and of the carotid flow

(*cf* Brown and Brierley 1971) If it is further assumed that these areas are totally ischemic and therefore electrically silent both the deranged energy state and the EEG pattern may be explained If confirmed the experiments thus corroborate the current belief that ischemia and not hypoxia *per se* is the pathophysiological factor which underlies serious derangement of the energy metabolism of the brain (see Siesjo and Plum 1972)

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Comparison between the Membrane Phases of the Catecholamine Storage Vesicles from the Adrenal Medulla and the Sympathetic Nerve Trunk

By

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The catecholamine storing vesicles of the adrenal medulla (= A vesicles) and the splenic nerve trunk (= N vesicles) have a number of specific proteins in common the high potential cytochrome b_{61} (Flatmark *et al* 1971) chromogranin A (Helle *et al* 1971) and chromomembrin B (Asamer *et al* 1971) The A and N vesicles differ significantly in size and ratio of membrane to matrix phases and the N vesicles are more stable with respect to the NADH (acceptor) oxidoreductase activities The effects of a neutral detergent on the hydrophobic interactions between lipids and proteins in the membrane containing phases will now be reported

N vesicles were obtained from homogenates of bovine splenic nerve trunks by differential and density gradient centrifugation (Klein and Lagercrantz 1971) The gradient fractions were diluted with equal volumes of 5 mM Na succinate pH 6 0 and centrifuged for 55×10^5 g min The pellet was washed twice in 10 vol buffer and centrifuged as before The pellet of water insoluble (WIS) protein was used as the source of membrane containing protein

A vesicles were obtained from bovine adrenal glands and lysis carried out by dialysis against 5 mM Na succinate pH 6 0 (Helle 1971) The soluble protein was removed by centrifugation at 55×10^5 g min The twice washed pellet of WIS protein was taken as the source of membrane phase protein

Solubilization in Triton X 100 (Triton) Aliquots of protein was diluted to a final concentration of 0 6—0 8 mg/ml in volumes of 250 µl of Triton concentrations ranging 0 5—20 % (v/v) in 170 mM NaCl The suspensions were left for 1 hr on ice before centrifugation at 4×10^5 g min The clear supernatants were assayed for total protein by the Folin phenol method and for chromogranin A by immunodiffusion Disc electrophoresis was carried out on 30—60 µg protein samples in 7 5 % polyacrylamide gels at pH 8 9 with 3 mA/gel for 1 h

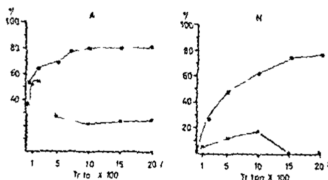


Fig 1 Effect of Triton X 100 on the solubilization of total protein and of immunologically active chromogranin A from the membrane phases of A and N vesicles Washed preparations of the membrane phases (WIS protein) were suspended in a series of concentrations of Triton X 100 in 170 mM NaCl The supernatants containing up to 0 8 mg protein/ml were assayed for total solubilized protein (●—●) and for solubilized immunologically active chromogranin A (■—■) given as % of the total membrane phase protein in each experiment

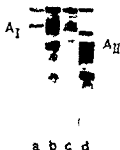


Fig 2 Disc electrophoresis of protein components of the membrane phases of A and N vesicles The patterns were obtained by polyacrylamide disc electrophoresis at pH 8 9 in 7 5 % gels with 3 mA/gel for 1 hr The samples were a) and b) A vesicle membrane phase protein solubilized by 1 and 5 % Triton X 100 respectively c N vesicle membrane phase protein solubilized in 5 % Triton X 100 d) "Soluble chromogranins (SM) of A vesicle

As shown in Fig 1 WIS protein of the N vesicle was more resistant to Triton than that of the A vesicle and the bound chromogranin A of the former was only partially solubilized with 1 % Triton used in previous estimations (Helle *et al* 1971) With 10 % Triton chromogranin A accounted for 12 1 ± 3 5 % of WIS protein which again represent about 80 % of the total N vesicle protein These results thus indicate that in the N vesicle the majority of the antigenic sites of chromogranin A is embedded in hydrophobic areas of the WIS protein structure in contrast to that observed for the A vesicle (Fig 1) for which 2 % Triton was sufficient to unmask the maximal amount of antigenic sites of the membrane phase chromogranin A The reduction in titre for the A vesicle with higher Triton concentrations may be an indication of hydrophobic interactions also between subunits of chromogranin A in the largest soluble aggregates notably A_I (Helle 1971)

The electropherograms obtained (Fig 2) show that A_I was the conspicuous component in the A vesicle at 1 % Triton (Fig 2 a) while A_{II} also could be seen together with faster moving components when 5 % Triton was employed (Fig 2 b) thus resembling the pattern obtained with the soluble matrix phase (Fig 2 d) This effect of increasing Triton concentration should outrule the suggestion (Winkler *et al* 1971) that A_{II} in the WIS protein be a trace contamination of the matrix phase

A close similarity can be seen in the electrophoretic patterns of the A and N vesicle WIS protein (Fig 2 b and c) when solubilized in 5 % Triton indicative of apparent identity also of the aggregates of molecular sizes larger than A_I and A_{II}

The N vesicle preparation used have been found to be more than 50 % pure with respect to NA containing vesicles (Klein and Thureson—Klein 1972 personal communication) a finding which allows for a comparison of the WIS protein of this preparation with that of the highly purified A vesicles even if the values for the N vesicles may have to be corrected when better preparations become available

The observed dissociative effects of Triton X 100 on WIS protein of the N and A vesicles have made it evident that hydrophobic interactions between lipid and protein are more important in the N than in the A vesicle, also in the binding of chromogranin A in these membrane containing phases Differences in response to changes in osmolarity and to prolonged storage on the catecholamine store and on enzymic activities may thus in part be related to the occurrence of stronger hydrophobic interactions between lipid and protein in the N than in the A vesicle

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Prostaglandin E Restricting Noradrenaline Secretion —Neural in Origin?

By

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It has not been known exactly how or from what source the prostaglandin E (PGE) involved in the control of noradrenaline (NA) secretion from sympathetic nerves (*cf* Hedqvist 1969) is released Contraction of the sympathetically denervated spleen induced by infusion of adrenaline leads to release of PGE (Gilmore Vane and Wyllie 1968) PGE release in the spleen is prevented by *α* adrenoceptor blockade (Davies Horton and Withrington 1967) This suggests that NA released by sympathetic nerve stimulation may trigger PGE release by stimulating postjunctional *α* adrenoceptors thus inducing muscular contraction However there is also the alternative that the very process of NA secretion from sympathetic nerves is the factor which triggers the release of PGE from the nerves themselves The nerves might thus concomitantly release transmitter to control postjunctional effectors and a substance with the function to terminate the secretory process

In the experiments reported in this paper the isolated superfused field stimulated guinea pig vas deferens was used to study the nerve stimulation induced secretion of NA when muscular contraction was prevented by using low calcium concentrations in the medium Blocking of PGE formation by ETA markedly enhanced NA secretion suggesting that PGE mediated control of NA secretion may represent a prejunctional feed back control mechanism independent of postjunctional events

The experiments were carried out in 14 guinea pigs weighing about 250 g The details of the technique used have been presented elsewhere (Stjarne 1973) NA secretion was monitored by determination of the nerve stimulation induced rise in efflux of total ^{3}H after preincubating the tissue with 10 μCi/ml of ^{3}H l NA (New England Nuclear Corp specific activity 6 5 Ci/mmol) Desipramine 6×10^{-7} M and normetanephrine 10^{-5} M were present in the superfusion medium Tyrode solution in order to block reuptake of NA (*cf* Iversen 1967) Nerve stimulation was performed by field stimulation with biphasic pulses of near maximal voltage and a duration of 1 5 ms Trains of 300 stimuli were applied at 5 Hz with about 10 min intervals between stimulation periods Contractions were recorded by means of an isotonic transducer (Harvard) loaded to balance the resting tone of the preparation recordings were made on a Grass Polygraph

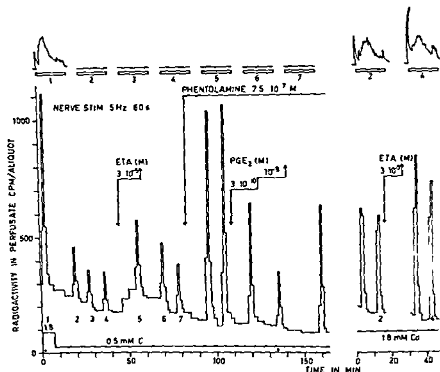


Fig 1 Nerve stimulation induced contraction (upper panels) and efflux of ^3H (lower panel) Desipramine and normetanephrine present throughout *Left panel* Effect of ETA to block PGE synthesis at low calcium Effect of exogenous PGE during α receptor blockade *Right panel* Effect of ETA at normal calcium

Fig 1 shows the results of a typical experiment Reducing the calcium concentration of the Tyrode solution from 1 8 mM to 0 3 —0 5 mM completely eliminated the contractile response to nerve stimulation and reduced the rise in efflux of ^3H to an average of 19 5±4 1 % (n = 7) of that resulting from the preceding control nerve stimulation at 1 8 mM calcium Infusion of ETA 3×10 M a concentration known to irreversibly and almost completely block PGE synthesis (Downing Ahern and Bachta 1970) frequently raised the resting efflux of ^3H particularly at low calcium Nerve stimulation during and after ETA infusion enhanced the ^3H efflux above baseline by 161 6±11 1 % (n = 14) when compared to the stimulation immediately before ETA Infusion of ETA at normal calcium 1 8 mM enhanced the nerve stimulation induced contractile response and raised the efflux of H over baseline by 146 9+4 7 % (n = 15) Even at low calcium the α adrenoceptor blocking drug phentolamine markedly elevated the nerve stimulation induced efflux of ^3H Infusion of PGE against this elevated baseline showed that the secretion of NA becomes very sensitive to PGE at low calcium the ID_{50} at 0 3—0 55 m calcium is 3 5±0 4×10^{-10} M (n = 7 cf Stjarne 1972)

The experiments consistently show that exposure to the inhibitor of PGE synthesis ETA causes a marked rise in nerve stimulation induced secretion of tracer and therefore most probably of total endogenous NA. This is the case even when the contractile response is completely prevented by low calcium. The results are consistent with the possibility that the PGE involved in control of NA secretion is of neural origin. It seems probable that PGE is released from the nerves in proportion to the amount of transmitter secreted. Judging from the NA release in the present experiments the amount of neural PGE released at low calcium may have been about 1/5 of that released at normal calcium since the sensitivity to PGE at low calcium is elevated about ten fold (Stjarne 1972) this low amount of PGE apparently was about as effective in restricting NA secretion as the larger amount of PGE released at higher calcium concentrations

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